University of Alberta

Pathogenesis of Post-operative Crohn's Disease Recurrence

by

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ABSTRACT

The recurrence of Crohn's disease following ileocecal resection is a common and serious problem. The aim of this thesis is to characterize changes in the neo-terminal ileum after ileocolonic anastomosis using IL-10 gene deficient mice prone to develop Crohn's disease. Ileocolonic anastomosis resulted in altered bacterial profile in the neo-terminal ileum and an increase in mucosal expression of TNF and TGF-β mRNA as well as systemic IFN-γ and IL-17 in response to endogenous bacterial antigens. Elevated mucosal TGF-β mRNA coincided with increased collagen deposition in the bowel wall of the neo-terminal ileum. On histology there was no mucosal inflammation, however, significant infiltration of mesenteric fat with lymphocytes and neutrophils was noted. Net glucose transport in the small bowel was not affected by ileocolonic anastomosis. This project demonstrated that a number of changes occur following ileocolonic anastomosis that may play a role in post-operative disease recurrence.

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LIST OF ABBREVIATIONS

3-*O*-Methyl-D-glucose	3-OMG
5-aminosalicylates	5-ASA
6-mercaptopurines	6-MP
Azathioprine	AZA
Bovine serum albumin	BSA
Coefficient of similarity	C_s
Conductance	G
Control animals: no operation	C
Crohn's disease	CD
Denaturing gradient gel electrophoresis	DGGE
Enzyme-linked immunosorbent assay	ELISA
Experimental operation	E
Fluorescence in situ hybridization	FISH
Hematoxylin and eosin	H&E
Immunoglobulin A	IgA
Inflammatory bowel disease	IBD
Inteferon gamma	IFN-γ
Interleukin-1	IL-1
Interleukin-6	IL-6
Interleukin-8	IL-8
Interleukin-10	IL-10
Interleukin-10 gene deficient or knockout mouse	IL-10 KO
Interleukin-12	IL-12
Interleukin-17	IL-17
Interleukin-23	IL-23
Irritable bowel syndrome	IBS
Lamina propria	LP
Major histocompatability complex II	HC II
Messenger ribonucleic acid	mRNA
Nuclear factor kappa B	NF-κB
Nucleotide-binding oligomerization domain 2	NOD2

Pathogen associated molecular pattern	PAMP
Pattern recognition receptor	PRR
Polymerase chain reaction	PCR
Polymerase chain reaction denaturing gradient gel electrophoresis	PCR-DGGE
Polymorphonuclear	PMN
Post-operative day	POD
Potential difference	PD
Prostaglandin	PGE
Randomized controlled trial	RCT
Relative quantification real time polymerase chain reaction	qRT-PCR
Reverse transcription PCR	RT-PCR
Ribosomal ribonucleic acid	rRNA
Sham operation	S
Short circuit current	I_{sc}
Small bowel obstruction	SBO
Specific pathogen free	SPF
Standard deviation	SD
Standard error of the mean	SEM
Terminal ileum	TI
T helper 1	Th1
T helper 17	Th17
Toll-like receptor	TLR
Transforming growth factor beta	TGF-β
Tumor necrosis factor	TNF
Ulcerative colitis	UC
Wild type	WT

Chapter 1. Surgical Management of Crohn's Disease

1.1. Crohn's Disease

Crohn's disease (CD) is a chronic inflammatory gastrointestinal disorder. Although the disease most frequently involves the terminal ileum, it can affect any portion of the gastrointestinal tract from the mouth to the anus. At diagnosis, 47% of patients have disease localized in the terminal ileum and 21% have involvement of the ileocolon.[1] CD is most prevalent in North America and Europe, with an estimated occurrence of 0.2% in the United States and central Canada, and peak incidence in the second decade of life.[2, 3] The etiology of CD remains unclear, and both basic science and clinical research suggest the etiology is multifactorial. A combination of genetic predisposition, multiple bacterial agents and environmental factors most likely results in disease onset and progression.

There is no cure for CD and the goal of medical and surgical therapies is symptomatic relief. Medical management is the first line of therapy with surgery reserved for patients who have refractory symptoms despite optimal medical management or who have severe adverse effects related to medical therapy. Surgery is also used for the treatment of complications such as obstruction, perforation, hemorrhage, enteric fistulas, dysplasia or cancer and growth retardation. The most common indications for surgery are failure of medical management and obstruction.[4] It has been estimated that 75% of CD patients will require surgery within 20 years of the onset of symptoms, many of whom will require subsequent operations.[5]

1.2. Surgical Management of Crohn's Disease

STRICTUROPLASTY

The goal of surgical therapy is to alleviate symptoms with limited or no bowel resection, and to minimize the risk of complications such as short bowel syndrome. The most commonly performed operations for CD are stricturoplasties and bowel resections. A stricturoplasty is a technique aimed at conserving bowel length where the lumen is effectively widened, without any bowel resection, at the site of the obstruction-forming stricture (Figure 1.1). This procedure is typically used for treatment of short segments of strictures in the jejunum and ileum.[6]

ILEOCOLONIC RESECTION

Forty to fifty percent of patients referred for surgery have disease of the terminal ileum.[4] Although, ileal or ileocolonic obstruction due to strictures can be treated with stricturoplasties, often the disease in this portion of the bowel requires resection due to the presence of a perforation, fistula or long strictured segment. An ileocolonic resection removes part of the terminal ileum, the cecum and a portion of the ascending colon. The small bowel is then re-connected to the large bowel in an end-to-end or more commonly a side-to-side anastomosis (Figure 1.2). The estimated probability of ileocolonic resection after an average of 13 years of disease activity in the terminal ileum is 91.5%.[7]

The ileocolonic resection also removes a sphincteric structure located at the junction of the small and large bowel, the ileocecal valve. The ileocecal valve is a thickened layer of circular muscle, which has been shown to have sphincter properties that facilitate forward movement of gut contents from ileum to colon and prevents reflux of colonic contents into the small bowel.[8] Although the bacterial diversity in the ileum resembles the colonic bacterial populations, the number of bacteria significantly increases from 10^7-10^8 bacteria/mL in the ileum to 10^{13}-10^{14} bacteria/mL in the colon, suggesting the presence of a physical barrier between the two compartments.[9] Manometric studies have shown an increased and sustained pressure at the ileocolonic junction of 10 mm Hg for a distance of approximately 5 cm.[10] Furthermore, there exists a mechanism within the distal ileum and ileocecal valve, involving myogenic and neuronal components, that effectively clears colonic reflux from the small bowel.[10] Loss of the ileocecal valve, as in the case of ileocolonic resection, causes functional changes at the ileocecal junction resulting in reflux of bacteria-rich colonic contents into the small bowel and most likely increased bacterial colonization of the neo-terminal ileum.

There is compelling evidence from both animal models of inflammatory bowel diseases (IBD) and human studies that the gastrointestinal commensal flora plays a pivotal role in the pathogenesis of CD and in the recurrence of the disease after ileocolonic anastomosis. CD occurs predominantly in the terminal ileum, cecum and colon, which are also the segments of the gastrointestinal tract with the highest bacterial counts.[11] Although no single microbial agent has been found to be the cause of CD onset and progression, multiple investigators have demonstrated changes in both the commensal flora diversity and enumeration.[12, 13,14, 15] Previous studies have shown that the absence or reduction of gut microbes prevents bowel inflammation or ameliorates it.[12, 13, 16-19] For example, under germ-free conditions, the majority of animal models of IBD do not develop intestinal inflammation.[16] When animals are returned to conventional housing, inflammation occurs. Exclusion of fecal contents from segments of bowel prevents inflammation in those segments but re-introduction of fecal stream results in rapid inflammatory changes.[12, 13, 17] In addition, post-operative antibiotic therapy results in the delay of disease recurrence and reduced severity.[18, 19]

1.3. Post-operative Recurrence of Crohn's Disease

Surgical management of CD offers immediate resolution of acute symptoms, however, surgical success tends to be short lived. The overall reported post-operative recurrence rates remain high despite advances in the medical treatment of CD with clinical recurrence of 17-55% at 5 years, 32-76% at 10 years and 70% at 20 years.[20] Furthermore, a considerable number of patients with recurrence of CD will require subsequent operations, which are more complex procedures and have an increased incidence of complications associated with repeated bowel resections. The estimated overall incidence of re-operation is 11-32% at 5 years, 20-44% at 10 years and 46-55% at 20 years.[20]

Both the site of the initial operation and the surgical procedure performed influences the pattern and rates of recurrence. A large meta-analysis of 1,112 patients undergoing 3,259 stricturoplasties (94% of cases) involving the jejunum and/or ileum revealed recurrence rates requiring re-operation in 14% of patients at 5 years.[6] In comparison, patients with surgically treated ileocolonic disease tend to have a higher frequency of re-operation at the same time interval (5 years) of 53%.[21] Interestingly, the site of recurrence amongst 90% of patients undergoing stricturoplasty within the jejunum and/or ileum was remote from the original site of operation.[6] In contrast, a retrospective study of 78 patients undergoing a re-operation for CD recurrence revealed recurrent disease at the previous anastomosis in 70% of bowel resections.[22] In addition, the disease recurred at the anastomosis in 83% of patients who had previously undergone ileocecal resection and inflammation had extended into the proximal limb of the neo-terminal ileum.[22]

For ileocecal resection, the pathophysiology leading to clinical symptoms appears to take place in the immediate post-operative period. Up to 72% of patients show endoscopic evidence of disease, such as aphthous ulcers, in the neo-terminal ileum in the first post-operative year, yet only 20% have symptoms of the disease.[23] Clinical follow up and endoscopy at 3 and 10 years show progressively more severe bowel wall disease and an increase in the number of patients experiencing symptoms of CD.[23] The knowledge of these early post-operative changes presents an opportunity for early intervention. Unfortunately, risk factors, pathogenesis and prophylactic measures for CD recurrence remain poorly defined and understood.

1.4. Risk Factors for Post-operative Recurrence of Crohn's Disease

Given the significant burden of recurrent CD on the health care system and more importantly on the patient, a considerable amount of clinical and basic research has been dedicated to identifying risk factors or predictors of disease recurrence. Clinical retrospective studies have explored numerous factors, which can be broadly classified as patient factors, disease factors, surgical factors and other factors.

PATIENT FACTORS

The contribution of patient factors such as age at disease onset, gender and use of oral contraceptive pills have been investigated, however, currently none of these factors are considered predictors of disease recurrence.[24-27] Conversely, a large volume of evidence supports smoking as an independent risk factor for endoscopic, clinical and surgical recurrence.[25, 27-32] A study of 174 patients demonstrated that 29% and 41% of non-smokers required a re-operation at 5 and 10 years respectively. The same study revealed that these rates were significantly higher amongst smokers - 36% at 5 years and 70% at 10 years.[30] Smokers also had a significantly higher risk of having up to three re-operations for recurrence at any site, however, this risk was significantly reduced, and the duration to re-operation was significantly increased if a patient quit smoking.[29]

DISEASE FACTORS

Disease associated factors that have been implicated in early post-operative recurrence include the duration of disease prior to initial surgical therapy and perforating disease. A prospective study following 233 patients over 15 years showed an increased probability of recurrence if initial surgery was performed within 69 months from the symptom onset.[33] This finding was further confirmed in retrospective studies in both adult and pediatric populations where post-operative recurrence was more likely if initial surgery was performed between 1 and 10 years after diagnosis.[34, 35] Despite this evidence, the relationship between duration of disease and recurrence remains inconclusive given that multiple other studies have failed to demonstrate an association.[24, 36, 37]

According to the Vienna classification, CD follows one of three behaviour patterns: (1) non-stricturing and non-perforating (70%), (2) stricturing (17%) and (3) perforating (13%).[1] Perforating CD is characterized by the development of acute free perforations, abscesses or fistulas, and appears to represent a more complex and aggressive form of disease resulting in earlier post-operative recurrence. A review of 770 patients showed that those with perforating disease were not only more likely to require re-operation for recurrent perforation, but also required re-operation two times earlier then patients with non-perforating disease.[38] The risk of re-operation for recurrence among patients with perforating disease has been shown to be the highest in the first 2 post-operative years.[39] Although a number of studies have failed to show a relationship between perforating disease and early recurrence, a recent meta-analysis of 13 studies and 3,044 patients showed a significantly increased risk of re-operation for patients with perforating disease.[33, 40-42] Furthermore, the type of disease requiring surgery also predicts the type of recurrent disease, meaning that perforating disease leads to perforating type of recurrence and non-perforating disease recurs as a non-perforating disease.[42]

SURGICAL FACTORS

The risk factors associated with surgical techniques have been explored in depth. Identification of these risk factors can facilitate changes in surgical procedures and techniques such that post-operative outcomes can be optimized. Factors such as the length of bowel resected, microscopically positive resection margins, number of resections, presence of granuloma in the resected specimen and post-operative complications have not been consistently shown to be predictive of disease recurrence.[26, 33, 43-47] Interestingly, the presence of myenteric plexus inflammation (myenteric plexitis) in the proximal margin of ileocolonic resections has been found to be an independent risk factor for early endoscopic recurrence at 3 months and 1 year.[48]

Most surgeons employ the minimalist philosophy in the surgical treatment of CD and prefer to remove grossly diseased bowel leaving behind margins with microscopic disease. In cancer operations, a successful operation is partially defined by the disease-free resection margins, which is not the case in CD. CD may involve prolonged segments of bowel and/or multiple sites, and as such, there is high likelihood that there will be a future need for re-operation. The resection of long segments of bowel (more then 50% of total length) and subsequent loss of functional bowel can lead to serious complications such as short bowel syndrome. Short bowel syndrome is a chronic malabsorptive state that can lead to life threatening electrolyte imbalances and chronic nutritional insufficiency leading to growth retardation in the younger patients.[49] The significance of disease-free resection margins has been studied extensively. Several studies support the concept that surgical removal of only gross disease has no impact on post-operative disease recurrence.[24, 33, 34, 47, 50-53] For example, in a prospective clinical trial of 152 patients undergoing ileocolonic resection, randomized to conservative resection (2 cm proximal margin) or radical resection (12 cm proximal margin), no difference in the post-operative recurrence rates were observed between the two groups.[54]

The recurrence at the site of anastomosis has been well described.[22] As a consequence, the anastomostic technique has been closely examined in a large number of studies looking specifically at the ileocolonic anastomosis.[25, 55-62] The anastomosis of small bowel to large bowel is of importance since the ileocecal resection is one of the most frequently performed operations for CD, and technically this anastomosis requires joining of segments of bowel of different luminal caliber and distinct physiological characteristics. The construction of the anastomosis involves a number of options for material used in the reconnection of the two bowel ends and different configurations (Figure 1.2). The two materials used for re-establishing bowel continuity are staples in a stapled anastomosis or a synthetic monofilamentous suture such as in a hand-sewn anastomosis. The three configuration options are end-to-end, end-to-side and side-to-side anatomoses. The material and configuration options can be used in a number of combinations, which makes a direct comparison between studies to determine optimal anastomotic selection challenging. A recent meta-analysis comparing stapled to hand-sewn ileocolonic anastomosis of any configuration for all causes (cancer and CD) demonstrated a decreased rate of anastomotic leak in the stapled group.[55] No sub-group analysis was performed for the CD population due to small sample size. In CD, when comparing hand-sewn to stapled end-to-end anastomoses there is conflicting evidence, with some studies demonstrating increased post-operative complications and recurrence rates with hand-sewn anastomoses while other studies failing to show any such association.[25, 56-58] Both the hand-sewn side-to-side and stapled side-to-side anastomoses have similar lower re-operative rates when compared to stapled end-to-side anastomosis, suggesting that the configuration rather than the material may play a more important role.[59] The superiority of side-to-side anastomosis in terms of lower post-operative leak and recurrence rates has been confirmed in a number of studies.[60, 61, 63] A proposed explanation for this observation is that the wide lumen formed by the side-to-side anastomosis prevents fecal stasis and excessive bacterial colonization of the anastomotic site, which most likely contribute to disease recurrence. However, the most recent meta-analysis confirming that the side-to-side anastomosis results in lower anastomotic leaks and lower overall post-operative complications did not provide sufficient evidence to support that this configuration has less frequent peri-anastomotic recurrence. [62]

A new advancement in the surgical management of CD is the introduction of laparoscopic surgery, specifically laparoscopic ileocecal resection. The safety and benefits of the laparoscopic approach in comparison to the standard open technique have been studied in depth. Although the laparoscopic approach, on average, takes longer to perform (mean 25 minutes longer), it has multiple benefits such as lower post-operative morbidity, faster gastrointestinal functional recovery, reductions in blood transfusions, post-operative narcotic use and length of hospital stay, in addition to a more desirable cosmesis.[64, 65] The disease recurrence rates are similar for the two approaches.[64] The described advantages of laparoscopy apply to select patients who are undergoing elective surgery and exclude patients with diffuse peritonitis, complex fistulae, dense adhesions and emergency operations. In experienced centers, laparoscopic surgery can be performed safely in these more complex cases without increased morbidity and mortality.[66]

OTHER FACTORS

Currently there are no recommended guidelines for post-operative recurrence prophylaxis or follow-up. The lack of standard post-operative care guidelines results from a poor understanding of the pathogenesis of post-operative CD, limited efficacious prophylactic interventions and an inability to reliably risk-stratify patients for recurrence. It is important to identify patients who are likely to have recurrent CD to provide them with necessary medical care. It is also of paramount importance to identify individuals who are not at risk for recurrence to avoid potentially lifelong toxic medical treatment, such as immunomodulators.

Multiple research groups have attempted to identify the predictors of recurrence from the radiological, genetic, and immunological perspectives and although there is no widely available screening test to predict recurrence, there have been some promising results. Studies have examined the bowel wall thickness, local and systemic production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-1β (IL-1β), the production of specific anti-microbial antibodies, and genetic variability in the *NOD2/CARD15* gene as predictors of future clinical relapse.[67-76]

Abdominal ultrasound is a desirable imaging technique, given its non-invasive nature, and it has been used in the context of CD to determine the maximum bowel wall thickness, the number of sites and the length of bowel wall thickening as a representation of diseased small bowel. Using this technique, patients with bowel wall thickness >7 mm were found to be more likely to require a primary operation within a year. [67] A long pre-operative segment of bowel wall thickening as well as unchanged or worsened wall thickness from the pre- to post-operative period and wall thickness of >6 mm at 12 months after surgery all carried a higher risk of clinical and surgical recurrence.[68, 69] These results are promising, however ultrasound imaging is notoriously operator-dependent, making this technique an unreliable screening tool for changes in small bowel, not only between institutions, but also between individual radiologists.

More evidence is becoming available to support the role of the innate immune system in the pathogenesis of the CD. Nevertheless, type-1 helper T cell (Th1) mediated adaptive immune response and the pro-inflammatory cytokines such as IL-1β, IL-6 and TNF still play an important role.[70] The mucosal secretion of IL-1β, IL-6 and TNF was shown to be increased in patients with CD even in the absence of obvious inflammation.[71] An elevated production of IL-1β and TNF by the lamina propria mononuclear cells, and high serum levels of IL-6 predicted an early recurrence in the setting of medical remission.[77, 78] In the setting of surgical remission, a small prospective study of 36 patients showed elevated ileal mucosal IL-6 level to be an independent predictor of future recurrence after ileal or ileocecal resection.[72] Decreased pre-operative ileal messenger RNA (mRNA) level of interleukin-10 (IL-10) was found to be a marker for early endoscopic recurrence.[72, 73]

The association between altered innate immunity and CD came from the discovery of mutations in the *NOD2/CARD15* gene, which encodes the nucleotide-binding oligomerization domain 2 (NOD2) protein involved in the cytosolic pattern recognition of bacterial peptidoglycan.[70] This protein plays a pivotal role in engaging both innate and adaptive immunity through activation of specific transcription factors following binding of bacterial muramyl dipeptide. Approximately one third of patients with ileal disease carry a mutated variant of this protein.[70] Furthermore, in prospective genotyping studies, *NOD2/CARD15* variants have been shown to be associated with more aggressive disease in the form of intestinal stenosis, with higher likelihood of early surgical intervention and subsequently higher rate of recurrence, as well as the need for re-operation.[74, 75] Other markers of more aggressive stricturing and perforating disease, requiring small bowel surgery, have been identified to be antibodies to select microbes such as *Pseudomonas fluorescence* (I2 antibody), *Escherichia coli* (outer membrane porin C), and *Saccharomyces cerevisiae* (oligomannan) and antibodies to bacterial flagellin (CBir1 antibody).[79, 80] The expression of I2 antibody was also predictive of disease response to surgical fecal diversion in the setting of severe colorectal and perianal CD.[76] The presence of multiple microbial antibodies amongst patients with CD also correlates with the presence of *NOD2/CARD15* variants, linking the involvement of both innate and adaptive immunity.[81]

CD is a multifactorial disease and it is unlikely that a single test will be sensitive enough to screen for post-operative recurrence. Instead, a panel of tests targeting the key characteristics of different phenotypes of CD may eventually identify individuals with the most aggressive disease and thus, at the highest risk of surgical interventions and post-operative recurrence. These individuals are most likely to benefit from close surveillance and prophylaxis.

1.5. Management of Post-operative Crohn's Disease

Currently the available post-operative prophylactic therapy has only minimal efficacy based on prospective studies. The conventional agents used in the medical induction and maintenance of remission have been tested in the post-operative setting with limited success.The agents evaluated for post-operative prophylaxis include: 5-aminosalicylates (5-ASA), immunomodulators, steroids, antibiotics, probiotics, monoclonal antibodies (anti-TNF antibody) and IL-10 (Table 1.1).

5-AMINOSALICYLATES (Table 1.2)

5-ASA is a class of anti-inflammatory agents that decrease local bowel inflammation and includes sulfasalazine and mesalamine, which can be formulated to target specific gastrointestinal sites. There is very weak evidence for sulfasalazine's effectiveness in post-operative recurrence. Two small randomized controlled trials using sulfasalazine at 5 g/day and 3 g/day versus placebo showed no difference in clinical recurrence at 1 year.[82, 83] A large multi-centre trial of 232 patients receiving daily sulfasalazine (3 g/day) or placebo showed reduction in the endoscopic, clinical and radiological recurrence rates in the treatment group at 1 year, however no difference was observed at the 3 year follow up.[84]

The use of mesalamine for operative CD remission maintenance has received the most attention. There have been eight randomized controlled trials, seven of which compared mesalamine to placebo and one that compared it to 6-mercaptopurine (6-MP).[85-92] The results of these studies are discordant. The first two moderately sized studies of 110 and 163 patients, using 2.4 g/day and 3 g/day of mesalamine respectively versus placebo showed positive effects. The patients using 2.4 g/day had lower rates of endoscopic and symptomatic recurrence at 2 years and patients using 3 g/day followed for up to 6 years also had lower symptomatic recurrence.[85, 86] Two subsequent studies did not show any statistically significant results in favour of mesalamine, however, one study of 87 patients on 3 g/day of mesalamine had less severe lesions on endoscopy at 12 months.[87-89] Despite these findings, a meta-analysis summarizing the five studies still demonstrated a 13% recurrence reduction for patients on mesalamine.[93] The three randomized controlled trials that followed the meta-analysis diluted the previously reported benefits of mesalamine. One of the trials, being the largest multi-centre trial to date, of 318 patients receiving 4 g/day of mesalamine versus placebo, did not demonstrate a difference in the recurrence rates between the two groups at 18 months.[90] However, subgroup analysis showed lower relapse rates amongst patients on mesalamine who had disease isolated to the small bowel. [90]

The variable results between studies have been attributed in part to different regiments of mesalamine between trials. A recent systematic review pointed out the use of different formulations of mesalamine in the different studies, such as pH-dependent and controlled-release mesalamine, which allow the drug to target different sites in the gastrointestinal tract.[94] The pH 7 mesalamine formulation, which targets the ileum and was used in one of the randomized controlled trials, resulted in the lowest post-operative relapse rates compared to placebo.[94] Further clinical trials using this specific formulation of mesalamine are needed to delineate the role of mesalamine in maintenance of surgical remission.

IMMUNOMODULATORS (Table 1.3)

The immunomodulators 6-MP and azathioprine (AZA) are purine analogues that interfere with DNA, RNA and protein synthesis, resulting in global down-regulation of the immune system. A number of small retrospective studies have suggested a benefit of AZA in lowering post-operative endoscopic recurrence, suggesting randomized prospective studies to validate such benefit.[95, 96] Only one randomized controlled trial has been conducted to date comparing 6-MP (50 mg/day) to mesalamine (3 g/day) and placebo.[92] Despite demonstrating that 6-MP was more effective in reducing post-operative endoscopic and clinical recurrence then mesalamine and placebo at 2 years, these results should be evaluated with caution.[92] The study was criticized for overestimating clinical relapse in the placebo group, a 20% drop out rate, lack of definition of the primary outcome measure and flawed statistical analysis.[97] An open label prospective trial comparing AZA (2 mg/kg/day) to mesalamine (3 g/day) did not reveal a difference in the clinical or surgical recurrence rates at 2 years in the two groups of patients following conservative surgery for CD.[98] However, in a subgroup analysis, patients on AZA who had previous bowel resection had lower relapse rates.[98] The potential for AZA and 6-MP to benefit patients with multiple bowel surgeries was shown in a small retrospective study where patients treated with the immunosupressants were less likely to undergo a third intestinal resection.[99]

STEROIDS (Table 1.4)

Steroids also fall within the immunosuppressive class of medications. Steroids play a pivotal role in the medical induction of remission in CD, however they have a limited role in medical remission maintenance, primarily due to their significant side effect profile that includes hyperglycemia, osteopenia and growth retardation in young patients. There is also no role for oral and topical formulations of steroids in surgical remission maintenance based on the results of two randomized prospective studies.[100, 101]

ANTIBIOTICS AND PROBIOTICS (Table 1.5)

The commensal gastrointestinal bacteria have been implicated in the pathogenesis of CD. Interventions such as antibiotics and probiotics that can modify gut flora have a potential to benefit CD patients and both have been evaluated in maintaining surgical remission.[12, 13, 16, 17] Two randomized controlled trials examining the role of antibiotics have been conducted. In the first study, 60 patients were randomized immediately after ileocolonic resection to metronidazole (20 mg/kg) or placebo for 3 months. Patients receiving the antibiotic were found to have significantly less severe endoscopic disease at 12 weeks post-operatively and lower recurrence rates at 1 year but no improvement in relapse rate at 2 and 3 years, suggesting that antibiotics delay the post-operative recurrence.[18] Ornidazole (1 g/day) was also better than placebo in a randomized controlled trial of 80 patients, where the treatment group had significantly lower post-operative endoscopic and clinical recurrence at 12 months after ileocolonic resection.[19] Despite these positive findings there was a high dropout rate in both studies in the treatment groups due to adverse side effects of treatment, including metallic taste and gastrointestinal upset.[18, 19]

Probiotics are non-pathogenic bacteria that when present in the gastrointestinal tract can improve the intestinal microbial balance. Studies in recent years on probiotics have revealed a variety of mechanisms through which these microbes benefit the host. These mechanisms include modulation of epithelial cell barrier function and epithelial cytokine secretion as well as anti-bacterial effects through secretion of anti-microbial peptides and competition with other microbes for colonization.[102] To date, five randomized controlled trials have not supported the use of probiotics in the prevention of the post-operative recurrence.[103-107]Endoscopic recurrence was not prevented at 1 year after bowel resection by a 12 month administration of *Lactobacillus* GG or at 3 and 6 months in patients receiving *Lactobacillus johnsonii* LA1.[103-105] Similarly, a combination of pre- and probiotics (Synbiotic 2000) did not result in improved endoscopic injury scores at 3 and 24 months after surgery.[106] A cocktail of probiotics (VSL#3) also failed to show endoscopic improvement at 3-month after surgery, however, it did have a down-regulating effect on the mucosal pro-inflammatory cytokine expression.[107]

OTHER STRATEGIES

Other novel strategies for maintenance of surgical remission include administration of recombinant human IL-10 (Tenovil) and anti-TNF antibody (infliximab). IL-10 deficient mice serve as an animal model for CD and as previously mentioned, low levels of ileal IL-10 mRNA at the time of surgery predicted early recurrence.[73] However, a randomized controlled trial of 58 patients who received subcutaneous Tenovil after ileocolonic resection did not demonstrate benefit in preventing endoscopic recurrence at 12 weeks.[108]

The evidence for the use of infliximab in the post-operative setting is based on very limited experience of one case report and a very small non-randomized prospective study, both from the same centre. [109, 110] In the case report, a 23 year old female remained free of endoscopic and clinical recurrence at 4 years post sigmoid resection while on a combination treatment of intravenous infliximab (5 mg/kg) every 8 weeks and weekly low dose of oral methotrexate (10 mg/week).[109] Following the same regimen, seven patients after various surgical interventions (ileal, ileocolonic and sigmoid resections) had no endoscopic or clinical recurrence at 2 years after surgery in comparison to a control group of 16 patients receiving daily mesalamine (2.4 g/day) who had a 75% recurrence rate at similar follow-up.[110]

Despite the ongoing efforts there remains a lack of sound and evidence-based guidelines for the surveillance and prophylaxis of CD patients following surgery. Numerous published reviews advocate smoking cessation and the use of mesalamine given its modest benefit in prospective studies.[111-113] In addition, based mainly on retrospective studies, many clinicians promote the use of immunomodulators (AZA and 6-MP) in patients deemed high risk for recurrence (patients with >1 operation, perforating or fistulizing disease).[113] Given the limitations of current prophylactic options yet persistently high post-operative recurrence rates, ongoing research in the area is of paramount importance to understand the pathophysiology that drives the recurrence and in hope that in the future this understanding will yield highly efficacious prophylactic measures.

1.6. Project Summary and Hypothesis

CD affects a significant number of North Americans and Europeans, typically at a young age, setting up a potential for a life long illness and poor quality of life. Despite the advances in research and new therapeutic options, such as monoclonal antibodies, CD often requires surgery. Despite its utility, surgery sets the stage for a vicious cycle of recurrent disease and subsequent re-operations. The ileocecal resection and ileocolonic anastomosis is one of the most frequent surgical interventions performed in managing severe terminal ileum and ileocolonic disease. This operation has the highest recurrence rates, with disease recurring at the anastomosis and in the neo-terminal ileum. The pathological changes appear to take place in the immediate post-operative period.

We hypothesize that the ileocecal resection removes the ileocecal valve, which leads to reflux of bacteria-rich colonic contents into the small bowel. This in turn leads to increased colonization of the small bowel by bacteria and provides an ideal environment for disease recurrence in a susceptible individual. The bacterial changes evoke a cascade of events from local and systemic immune changes to functional changes, and ultimately, tissue injury leading to inflammation. This translates into early endoscopic changes observed in the first post-operative year and subsequent clinical and operative CD recurrence.

Figure 1.1. Example of small bowel stricturoplasty

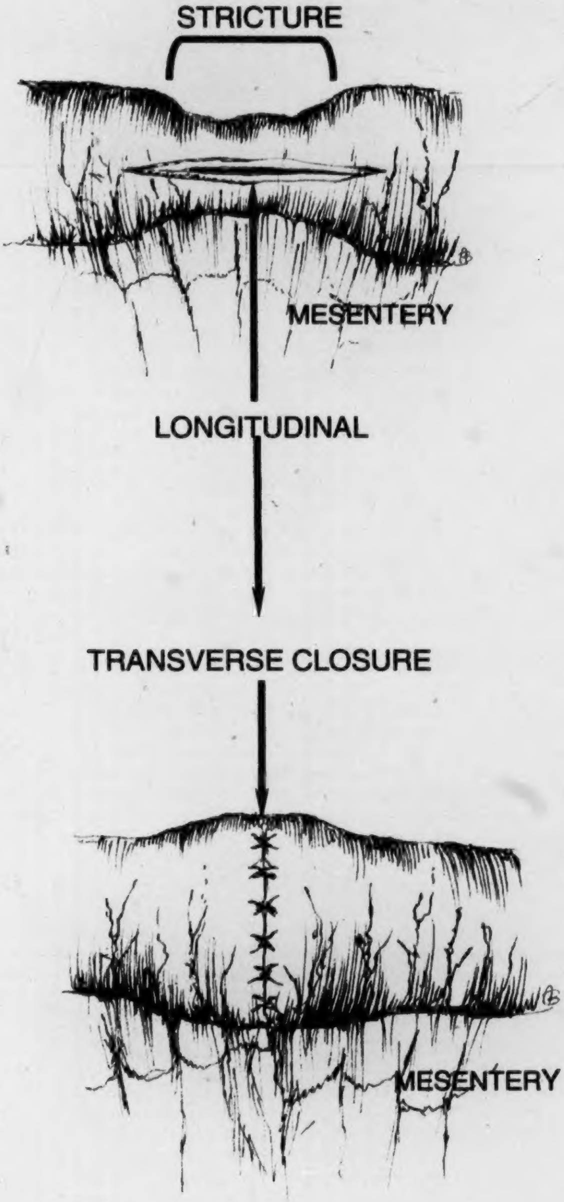
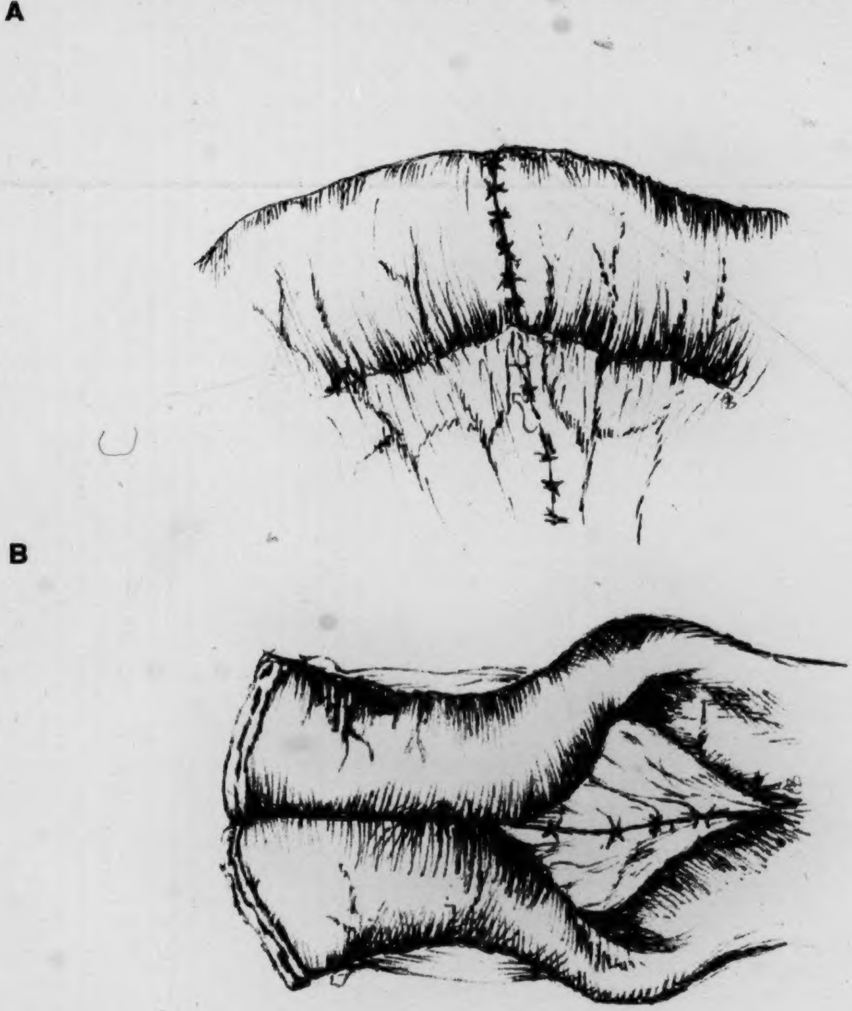

STRICTURE
MESENTERY
LONGITUDINAL
TRANSVERSE CLOSURE
MESENTERY

Figure 1.2. Configurations of bowel anastomosis



A. Hand-sewn end-end-to-end anastomosis. **B.** Stapled end-to-end anastomosis.

Table 1.1. Medications evaluated for prophylaxis of post-operative Crohn's disease recurrence

Medication group	Mode of action	Example used in CD
5'aminosalicylates	Local control of inflammation (exact mode unknown) and systemic inhibition of PGE synthesis	Sulfasalazine Mesalamine
Immunomodulators	Systemic immune down-regulation: antagonize purines (inhibit DNA, RNA, protein synthesis)	Azathioprine 6-Mercaptopurine
Steroids	Systemic immune down-regulation: suppress PMN migration, reduce activity and volume of lymphatic sustem	Prednisone Budesonide (topical)
Antibiotics	Inhibit bacterial growth (bacteriocidal or bacteriostatic)	Metronidazole Ciprofloxacin
Probiotics	Non-pathogenic bacteria that when present in the intestines they improve microbial balance, multiple mechanisms	*Lactobacillus* strain VSL#3
Monoclonal antibodies: anti TNF antibody	Inhibits a pro-inflammatory cytokine *TNF) by binding its receptor	Infliximab
Cytokines: IL-10	Anti-inflammatory cytokine	Tenovil

CD = Crohn's disease; PGE = prostaglandin; PMN = polymorphonuclear cells; TNF = tumor necrosis factor; IL-10 = interleukin 10.

Table 1.2. Summary of evidence for use of 5-aminosalicylates in prevention of post-operative recurrence of Crohn's disease

Study	Level of evidence	N	Treatment	Primary outcome	p-value
Wenckert *et al.* 1978	RCT	66	Sulfasalazine 3 g/day *vs.* placebo	Clinical at 1y	NS
Summers *et al.* 1979	RCT	28	Sulfasalazine 5 g/day *vs* placebo	Clinical at 1y	NS
Ewe *et al.* 1989	RCT	232	Sulfasalzine 3 g/day *vs* placebo	Endoscopic, radiological, clinical at 1y	<0.01
Caprilli *et al.* 1994	RCT	110	Mesalamine 2.4 g/day *vs* placebo	Endoscopic, clinical at 2y	<0.05
McLeod *et al.* 1995	RCT	163	Mesalamine 3 g/day *vs* placebo	Clinical at 6y	0.03
Brignola *et al.* 1995	RCT	87	Mesalmine 3 g/day *vs* placebo	Endoscopic, clinical at 1y	NS
Florent et al. 1996	RCT	126	Mesalamine 3 g/day *vs* placebo	Endoscopic at 3m	NS
Sutherland *et al.* 1997	RCT	66	Mesalamine 3 g/day *vs* placebo	Clinical at 1y	NS
Lochs *et al.* 2000	RCT	318	Mesalamine 4 g/day *vs* placebo	Clinical at 18m	NS
Caprilli *et al* 2003	RCT	165	Mesalazine 4 g/day *vs* 2.4 g/day	Endoscopic at 1y	NS
Hanauer *et al.* 2004	RCT	131	Mesalamine 3 g/day *vs* 6-MP 50 mg/day	Endoscopic, radiological, clinical at 2y	NS*

Primary outcome refers to recurrence rates (endoscopic, radiological, clinical, surgical). RCT = randomized controlled trial; y = year; m = month; 6-MP = 6-mercaptopurine; NS = not significant ($p > 0.05$); NS* = study by Hanauer *et al.*, outcomes were not significant for mesalamine and placebo groups only.

Table 1.3. **Summary of evidence for use of immunomodulators in prevention of post-operative recurrence of Crohn's disease**

Study	Level of evidence	N	Treatment	Primary outcome	p-value
Hanauer *et al.* 2004	RCT	131	6-MP 50 mg/day *vs* mesalamine 3 g/day	Endoscopic, radiological, clinical at 2y	<0.05
Ardizzone *et al.* 2004	Open label, prospective	142	AZA 2 mg/kg/day *vs* mesalamine 3 g/day	Clinical at 2y	NS

Primary outcome refers to recurrence rates (endoscopic, radiological, clinical, surgical). RCT = randomized controlled trial; y = year; AZA = azathioprine; NS = not significant (p > 0.05).

Table 1.4. **Summary of evidence for use of steroids in prevention of post-operative recurrence of Crohn's disease**

Study	Level of evidence	N	Treatment	Primary outcome	p-value
Bergman *et al.* 1976	RCT	97	Sulfasalazine with prednisolone *vs* placebo	Clinical at 1y, 2y, 3y	NS
Hellers *et al.* 1999	Open label, prospective	129	Budesonide 6 mg/day *vs* placebo	Endoscopic at 3m, 1y	NS

Primary outcome refers to recurrence rates (endoscopic, radiological, clinical, surgical). RCT = randomized controlled trial; y = year; m = month; NS = not significant (p > 0.05).

Table 1.5. Summary of evidence for use of antibiotics and probiotics in prevention of post-operative recurrence of Crohn's disease

Study	Level of evidence	N	Treatment	Primary outcome	p-value
Rutgeerts *et al.* 1995	RCT	60	Metronidazole 20 mg/kg/day *vs* placebo	Endoscopic at 3m	NS
Rutgeerts *et al.* 2005	RCT	80	Ornidazole 1 g/day *vs* placebo	Endoscopic, clinical at 1y	<0.05
Pantera *et al.* 2002	RCT	45	*Lactobacillus* GG	Endoscopic at 1y	NS
Marteau *et al.* 2006	RCT	98	*Lactobacillus johnsonii* LA1	Endoscopic at 6m	NS
Van Gossum *et al.* 2007	RCT	70	*Lactobacillus johnsonii* LA1	Endoscopic at 3m	NS
Chermesh let al. 2007	RCT	30	Synbiotic 2000 *vs* placebo	Endoscopic at 3m	NS
Madsen *et al.* 2008	RCT	120	VSL#3 *vs* placebo	Endoscopic at 3m	NS

Primary outcome refers to recurrence rates (endoscopic, radiological, clinical, surgical). RCT = randomized controlled trial; y = year; m = month; NS = not significant ($p > 0.05$).

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Chapter 2. Surgical Animal Model of Ileocolonic Anastomosis for Crohn's Disease

2.1. INTRODUCTION

Animal Models of Crohn's Disease

Animal models of human disease are essential to research as they allow researchers to answer crucial questions in settings where studies in humans are not possible. A large proportion of current knowledge on inflammatory bowel disease (IBD) pathogenesis and therapeutics has been derived from animal models. Animal models of IBD can be broadly divided into four categories: (1) spontaneous colitis, (2) inducible colitis models in an otherwise healthy wild-type background, (3) adoptive transfer models in an immunocompromised system and (4) genetically engineered models such as knockout and transgenic animals (Table 2.1).[1] Mice and rats are the two most common species of animals used for studies of IBD. Murine models are useful as their genome is fully characterized and as a result, genetic and immunological reagents for use in mice are readily available.[2] An ideal murine model of CD should mimic the pathology observed in humans without the use of genetic or immunological manipulation. This is not always the case as most of the mouse models of CD have disease localized to colon; exceptions are TNF ΔARE and SAMP1/Yit mice, which develop ileitis (Table 2.1).[3] Mice with disease limited to the colon are not truly representative of the human CD as the majority of patients have disease localized to the terminal ileum and ileocecum. Nevertheless, these models have greatly contributed to the current understanding of CD.

An example of a genetically modified mouse that develops CD-like colitis is the IL-10 gene deficient mouse, which we used in testing our project hypothesis.

Interleukin-10

IL-10 is a protein dimer that elicits cellular signal transduction by binding to a two chain transmembrane receptor that includes a cytoplasmic domain.[4] IL-10 is produced by a number of cells including B-lymphocytes, macrophages, thymic cells, and keratinocytes. The most significant producer of IL-10, especially in the setting of intestinal homeostasis, is a subset of CD4$^+$ regulatory T cells named Tr1.[5, 6] Tr1 cell differentiation is IL-10 dependent and the resulting T cells secrete high levels of IL-10, with variable levels of transforming growth factor beta (TGF-β) and interleukin-5 (IL-5).[6]

IL-10 has a multitude of functions with a key, unifying concept of being an anti-inflammatory cytokine. IL-10 was first described as an inhibitor of the T-helper 1 (Th1) pro-inflammatory cytokines interleukin-12 (IL-12) and tumor necrosis factor (TNF).[7] Other functions of IL-10 include inhibition of antigen presenting cells such as dendritic cells and macrophages, stimulation of CD8$^+$ T cells and finally, B-lymphocyte differentiation and immunoglobulin synthesis.[6, 8] The importance of IL-10 in intestinal homeostasis has been well described; high levels of IL-10 are produced by the regulatory T cells within the intestinal lamina propria, and genetically engineered mice lacking IL-10 develop gastrointestinal inflammation in the presence of bacteria.[9-11]

Interleukin-10 Gene Deficient Mice as a Model of Crohn's Disease

IL-10 knock out (IL-10 KO) mice were developed in 1993 by Kuhn *et al* and were described to have significant growth retardation (30% weight reduction in 75% of animals) and iron deficiency anemia in 90% of animals.[10] These findings were attributed to malabsorption secondary to intestinal inflammation, which under conventiona conditions in the original colonies described by Kuhn *et al* involved the entire gastrointestinal tract and was apparent at 4-8 weeks of age.[10] The most severely inflamed areas in these animals were the proximal colon, duodenum and proximal jejunum.[10] This inflammation is attenuated when animals are kept under specific pathogen free (SPF) conditions and absent under germ-free conditions, suggesting a role for microbes in the pathophysiology of bowel inflammation.[12,13] The observed inflammatory bowel changes in IL-10 KO mice include transmural lesions with hyper-regenerative and degenerative changes in the mucosa with abnormal crypt and villi architecture, thickened intestinal wall and erosions of the mucosa with inflammatory exudates, mucus and cellular debris.[10] Colorectal adenocarcinoma develops in a large number of animals at 6 months of age.[10, 11, 14] Upon further examination of this model, the IL-10 KO mouse was found to mount a chronic Th1 response characterized by an overproduction of pro-inflammatory cytokines IL-12, IFN-γ and TNF.[11] This pattern is also typical of CD in humans and thus further supports the use of IL-10 KO mouse as an animal model of CD.[8, 14] Since their development, IL-10 deficient mice have been studied extensively and they continue to provide valuable information on various aspects of CD. These aspects include bowel permeability, intestinal bacterial flora, and the cellular and molecular components of inflammation.[11-13, 15] This model has also served as a testing ground for potential therapeutic compounds such as anti-IFN-γ, anti-interleukin-12 (IL-12) and anti-TNF antibodies and IL-10.[14, 16-20]

The advantage of the IL-10 KO mouse as a model of CD is that it not only shares pathophysiological, but also immunological traits with the human disease. The IL-10 deficiency has contributed to the understanding of IL-10 function in health and disease.The main limitation of this model is the variability of disease severity between animals and under different housing conditions. Secondly, the artificially introduced immune deficiency (IL-10 deficiency) can make it difficult to attribute experimental results to a given intervention, as opposed to alterations in the underlying immune system. This limitation can be overcome by careful experimental design with appropriate use of controls. Thirdly, there is one exactly defined deficiency (IL-10 deficiency) that accounts for the disease development, which is not the case in human CD, which is multifactorial. Nevertheless, the IL-10 KO model used in conjunction with other animal models of CD can generate complementary knowledge that contributes to the understanding of CD pathophysiology and treatment.

The aim of our study was to establish an animal model of ileocolonic anastomosis in Crohn's disease that could then be used to examine any post-operative bacterial, immunological, functional and histological changes. An establishment of such an animal model has three prerequisites: (1) an animal with propensity to develop CD type intestinal inflammation, (2) the existence of an ileocecal valve at the mouse ileocecal junction, and (3) an operation mimicking an ileocolonic anastomosis performed in CD patients. To meet the first prerequisite we used the IL-10 KO mice described earlier. Second, since we were unable to find direct evidence for the existence of a mouse ileocecal valve in literature, we performed histological and functional examination of the ileocecal junction to confirm the presence of such a valve. Once the first two prerequisites were satisfied we then performed a wide side-to-side single layer ileocolonic anastomosis to meet the third prerequisite.

2.2. MATERIALS AND METHODS

Animals

Wild type (WT) mice were 129/SvEv from colonies maintained at the University of Alberta, [Edmonton, Alberta] under specific-pathogen-free (SPF) conditions and originally obtained from Taconic Laboratories [Hudson, NY]. The IL-10 KO mice were genetically modified mice deficient in the IL-10 gene, on a 129/SvEv background, from colonies originally obtained from DNAX Research Institute of Molecular and Cellular Biology Inc. [Palo Alto, CA] and maintained at the University of Alberta. Animals used for the experiments were WT and IL-10 KO males between 7 and 8 weeks of age. Single gender was chosen to eliminate the confounding effect sex hormones may have on gut bacterial populations and immune responses. In the presence of established bowel inflammation, IL-10 KO mice have a 30% mortality rate by 3 months of age.[10] We speculated this mortality would be further increased by surgical manipulation. Hence to increase the likelihood of post-operative survival we used animals 7 to 8 weeks of age. The IL-10 KO mice have minimal intestinal inflammation at this age. The animal ethics committee at the University of Alberta approved the use of animals for the experiments.

Three groups of animals were set up as follows, based on type of surgical procedure performed:

(1) Ileocolonic anastomosis operation group.

(2) Sham operation group.

(3) Control group (no operation).

Each group consisted of 18 WT and 18 IL-10 KO mice. The animals were analyzed at 6 and 15 post-operative weeks for bacterial flora changes, functional changes, local and systemic immune changes and histological changes. For the details of the analysis please refer to subsequent chapters as follows: bacterial changes in Chapter 3, functional changes in Chapter 4, local and systemic immune changes in Chapter 5, and histological changes in Chapter 6.

Ileocecal Valve

The existence of an anatomical and functional ileocecal valve in a mouse was determined by histological examination and cecal injections of methylene blue. For histology, IL-10 KO males and females as well as WT males and females in groups of six animals each were euthanized by cervical dislocation. Colon, cecum and small bowel were removed in continuity and the bowel was opened longitudinally displaying the ileocecal junction. A segment containing a portion of cecum and approximately 4 cm of terminal ileum was mounted onto a wax strip and fixed in 10% neutral buffered formalin [SF-94-4 Formalde-Fresh solution, Fisher Scientific, Fair Lawn, NJ] for 24 hours. Subsequently it was cut at 6 µm, stained with hematoxylin and eosin (H&E) and examined by single pathologist. The ileocecal junction was described in terms of thickness and length of the circular and longitudinal components of the muscularis propria, and presence of mucosal and submucosal folds at the ileocecal junction.

The function of the mouse ileocecal valve was assessed by 1 mL injection of 50% methylene blue [Omega, Montreal, QC] into the cecum tip. Four WT and 2 IL-10 KO male mice were anesthetized using inhaled isoflurane (2% isoflurane and 1 L/min oxygen). The abdomen was prepped with providone/iodine solution and the procedure was conducted under clean conditions. The abdominal cavity was entered through a small mid-abdominal longitudinal incision. The cecum was then delivered to the surface and 1 mL of 50% methylene blue was injected into the tip of the cecum under slow, gentle and constant pressure using a 30-gauge needle. The cecum was returned into the peritoneal cavity and the abdomen was closed in two layers (peritoneum/fascia and skin) using 6-0 braided silk sutures [ETHICON, Johnson and Johnson medical product, Markham, ON]. Animals received one dose of analgesic (buprenorphine 0.1 mg/kg) subcutaneously after closure. At 24 hours after the procedure the animals were euthanized by cervical dislocation and the entire bowel was removed. Both the colon and small bowel were opened longitudinally and examined under dissecting microscope for presence of methylene blue staining of the mucosa.

Surgical procedures

Ileocolonic anastomosis operation (Figure 2.1B)

Mice were fasted for 24 hours prior to surgery (receiving only water), and weighed on the day of the surgery. The operations were performed under an operating microscope. At the time of the procedure, animals were anaesthetized with inhaled isoflurane (2% isoflurane and 1 L/min oxygen) and positioned supine. The abdomen was cleaned with povodine/iodine solution and the remainder of the operation was carried out under clean conditions. A 1 cm longitudinal incision was made with scissors through the abdominal skin and fascia. The cecum was identified and delivered to the skin along with a generous segment of colon and terminal ileum.

A thin, filmy, avascular plane closest to the ileocecal junction was identified within the mesentery of the terminal ileum. This plane, which was usually within 1 cm of the ileocecal junction was divided by blunt dissection and became the point for terminal ileum transection. Prior to terminal ileum transection, a small artery running parallel and adjacent to the bowel at the point of transection was ligated with 10-0 nylon monofilament suture [*Sharpoint*, Surgical specialties corporation, Reading, PA] without causing any visible bowel ischemia. The terminal ileum was then tied off with 2 6-0 braided silk sutures [ETHICON, Johnson and Johnson medical product, Markham, ON] approximately 0.5 cm apart and the bowel was transected with scissors. A 3-5 mm enterotomy was made on the anti–mesenteric side of the proximal end of the transected ileum and a matching size enterotomy was made on the anti-mesenteric side of the ascending colon, 0.5 to 1 cm distal to the cecum. The two segments were then anastomosed in a side-to-side fashion (Figure 2.1A) with a single layer of interrupted 8-0 Prolene sutures [ETHICON, Johnson and Johnson medical product, Markham, ON].

The bowel was then returned into the abdominal cavity, which was filled with 0.5 to 1.0 mL of sterile 0.9% normal saline [0.9% sodium chloride, Abbott Laboratories, Montreal, PQ] and closed in two layers of running 6-0 silk suture. Immediately after closure, animals received 1-2 mL of 0.9% normal saline by subcutaneous injection; the volume was determined by the severity of intra-operative blood loss as estimated by the operator. Animals received one dose of subcutaneous analgesic (buprenorphine 0.1 mg/kg). In the immediate recovery period animals were kept in a separate cage on a heating plate on low setting and monitored frequently for signs of distress. Once there was adequate recovery as judged by ample movement, mice were returned to their cage and fed liquid diet [Rat Diet Control, BioServ, Frenchtown, NJ] for the first, post-operative 24 hours. At 24 hours after the operation, the standard solid diet was resumed [Laboratory Rodent Diet, Lab Diet, Richmond, IN].

Sham operation (Figure 2.1C)

Animals undergoing the sham procedure had identical pre-operative and post-operative handling as described above. The procedural steps such as anesthesia, abdominal incision and closure were conducted in the same manner as in the experimental animals. However, once the cecum was delivered to the skin the terminal ileum was transected 2 to 2.5 cm proximal to the ileocecal junction. The transected ends were re-anastomosed in an end-to-end fashion with 8-0 Prolene interrupted sutures over a digestible carbohydrate stent [Primo spaghettini, Kraft Canada] as described previously.[21]

Control (Figure 2.1A)

The control animals did not undergo any surgical manipulation. These animals were treated in the same manner as the animals that underwent surgery, including fasting overnight the day before surgery (receiving only water), weight measurement the day of the surgery, liquid diet for 1st, post-operative 24 hours and then return to the standard solid diet. In addition, control animals also underwent short induction of inhaled isoflurane anesthesia to perform ear notching for identification, and to control for the effects of anesthetic induction.

Mice in all groups were weighed daily for the duration of the experimental period and euthanized by cervical dislocation if displaying signs of distress (decreased movement, piloerection, or rectal prolapse), not gaining weight beyond the 4th post-operative day or at the set end points for the experiment (6 and 15 weeks after surgery). All animals euthanized prior to the experimental end points were replaced.

Statistical Analysis

Statistical analysis was performed using Stata 10.0 [Statacorp LP, College Station, TX]. Figures were generated using Excel Microsoft 2004 [Redmond, WA]. The ileocecal valve measurements were expressed as means ± SEM with two-sided p-values calculated using Mann-Whitney U test. The animal weights were reported as means ± SEM. Statistical analysis of weights was performed with ANOVA. If the null hypothesis of the ANOVA was rejected, then a multiple pair-wise comparison between groups was performed using unpaired Student t-test. To reduce type I error, Bonferroni's correction was applied where we considered a p-value to be statistically significant if it was less than 0.05 divided by the number of groups being compared.

2.3. RESULTS

Ileocecal Valve

The presence of an anatomical ileocecal valve was determined histologically and functionally. On gross morphology, the anatomical positioning of the terminal ileum and colon as they attach to the cecum is at 90 degrees. The histological examination of the H&E stained ileocecal junction in 24 animals, representing both the WT and IL-10 KO male and female mice, revealed a distinct mucosal fold at the transition from ileum to cecum (Figure 2.2). The fold was present in all 24 animals. Secondly, there was an obvious thickening of the smooth muscle making up the circular component of the muscularis propria (Figure 2.2). This thickening became apparent at the mucosal fold and extended into the cecum. The mean length of this circular muscle thickening was significantly longer in the IL-10 KO than the WT animals measuring 1.10 ± 0.11 cm and 0.59 ± 0.07 cm respectively ($p = 0.001$) (Table 2.2).

Injection of the cecum with methylene blue resulted in a preferential filling of the distal colon rather than the ileum suggesting the presence of a physical barrier at the ileocecal junction preventing reflux into small bowel. The functional evaluation at 24 hours after the injection of methylene blue into the cecum revealed there was no blue staining of the small bowel and colon. This finding is difficult to interpret as it may be due to antegrade movement of the dye and expulsion in the 24 hours without reflux into the small bowel or absorption of the dye by the bowel wall and renal excretion. The latter explanation fails to provide evidence for the existence of an ileocecal valve.

Mortality Rates

Overall, 62 WT mice and 64 IL-10 KO mice were included in the experiments (Table 2.3). Twenty and 21 animals underwent the sham operation in the WT and IL-10

KO groups respectively, and similarly 21 and 23 had the ileocolonic anastomosis operation. There were no intra-operative mortalities. There was no significant difference between the mortality rates in the WT and IL-10 KO groups (11.3% and 10.9% respectively). In both groups the highest mortality was observed amongst animals undergoing the ileocolonic anastomosis operation (22.7% for WT and 17.4% for IL-10 KO), followed by animals undergoing the sham operation (10.0% for WT and 9.5% for IL-10 KO).

The observed mortality can be classified as either early or late (Table 2.4). The majority of the animals (65%) had a necroscopy performed and the most frequent cause of death in the immediate post-operative period (1 to 7 days) was anastomotic leak. The second most common cause of early demise was obstruction at the anastomosis. Animals that died after the first post-operative week usually did so from causes unrelated to the anastomosis such as small bowel obstruction, intussusception and rare events such as aortic rupture.

Growth Curves

The IL-10 KO mice were significantly smaller than the WT mice at the start of the experiment with a mean weight of 17.27 ± 1.74 g *vs.* 21.21 ± 1.96 g ($p<0.001$) respectively (Figure 2.3). This significant weight difference was also observed at 6- and 15-week time points; IL-10 KO mice weighed 21.63 ± 1.71 g at 6 weeks and 23.56 ± 1.59 g at 15 weeks. At the same time points the WT mice weighed 27.68 ± 2.24 g and 29.92 ± 2.5 g ($p < 0.001$). The overall weight gain of 4.99 ± 1.58 g and 5.55 ± 1.30 g for the IL-10 KO mice over the 6 and 15-week post-operative periods respectively, was lower than the weight gain in the WT mice. The latter gained on average 6.07 ± 1.31 g and 8.64 ± 2.43 g ($p = 0.002$ at 6 weeks and $p < 0.001$ at 15 weeks). When WT and IL-10 KO mice were examined according to their treatment regime, there was no weight difference between the control, sham-operated and experimental animals at either time point (WT mice: at 6 weeks p = 0.681 and at 15 weeks p = 0.288; IL-10 KO: at 6 weeks p = 0.180 and at 15 weeks p = 0.515) (Figures 2.4 and 2.5).

2.4. DISCUSSION

The IL-10 KO mouse following ileocolonic anastomosis meets the following three criteria of a surgical animal model of CD: (1) an animal with a propensity to develop CD type intestinal inflammation, (2) the existence of an ileocecal valve at the mouse ileocecal junction, and (3) an operation mirroring the ileocolonic anastomosis. The IL-10 KO mouse is a well-established model of IBD and more specifically of CD. The bowel inflammation in IL-10 KO mice is well described in the literature as discussed earlier. In mice kept under SPF conditions, as is the case at the University of Alberta, this inflammation predominantly involves the colon and very rarely affects the proximal small bowel. For this reason, any changes in the ileum following an intervention such as surgery can be attributed to the intervention rather then the natural history of the disease.

The presence of an ileocecal valve in mice that can prevent free reflux of colonic contents into the small bowels is confirmed by both our results and previous research.[22] The mouse ileocecal valve is located at the junction of the terminal ileum and cecum and in very close proximity to the site where the cecum becomes the ascending colon. In fact, the insertion of the terminal ileum into the cecum is at 90 degrees to the colon and this spatial arrangement may be one of the factors contributing to the prevention of colonic reflux. At the epithelial transition from the ileal villi to the colonic crypts, there is a circumferential mucosal fold that can be readily identified on gross morphology and on histology. This fold may possibly function as a one-way valve further preventing movement of bowel contents in the retrograde direction. We also demonstrated a thickening of the smooth muscle layer of the bowel wall, a circular layer of the muscularis propria. Although not proven in mice, a similar thickening in smooth muscle in humans receives neuronal and myogenic input contributing to the sphincteric function of the human ileocecal valve.[23] Our data also demonstrated a significant difference in the length of this thickening between the WT and the IL-10 KO mice. This difference was not mitigated by the variability in the depth of the cuts during preparation of the slides as it was a consistent finding present in all IL-10 KO animals examined. It is likely that this thickening was an overlap with the diseased cecum in the IL-10 KO mice, where inflammation resulted in the bowel wall thickening.

We confirmed the presence of a functional barrier between the ileum and cecum by preferential filling of the distal colon rather than the terminal ileum following the injection of methylene blue into the cecum. Although this represents crude evidence of a functional ileocecal valve, additional compelling evidence for a functional ileocecal valve in mice comes from a study on the quantity and diversity of the bacterial populations on either side of the ileocecal junction.[22] Using fluorescence *in situ* hybridization, Swidsinski *et al.* showed that the ileum contains few, heterogeneous microorganisms, which are not in contact with the bowel wall.[22] In contrast, the mouse cecum was shown to contain a very high concentration of bacteria, too high to even quantify, and bacteria were in contact with the bowel wall.[22]

To meet the final requirement for a surgical model of CD, we devised an operation where the terminal ileum is transected and gastrointestinal continuity is then restored with a side-to-side ileocolonic anastomosis. Although no segment of bowel is removed, as is the case in the ileocecal resection in patients with CD, we argue that this is a sufficient procedure for the study of post-operative events in the neo-terminal ileum as this operation mimics the removal of the ileocolonic valve. The loss of ileocecal alone is sufficient as it is the pivotal mechanism that drives the reflux of colonic contents into the small bowel and we present the evidence for such reflux in a subsequent chapter (Chapter 3).

Our experimental and sham operations had an acceptable overall mortality rate of approximately 11% for both WT and IL-10 KO mice. Surprisingly there was no difference between WT and IL-10 KO mouse mortality, however, it should be mentioned that WT mice underwent surgery first in the course of the project during a steep learning curve for the operator. This may account for the high mortality rate observed in the WT animals. The highest mortality rate occurred among animals undergoing the ileocolonic anastomosis (22.7% in WT and 17.4% in IL-10 KO), which is a reasonable outcome given the more complex nature of this operation and longer operative time.

The predominant early post-operative causes of mortality were anastomotic leak and bowel obstruction at the anastomosis. These are well-recognized operative complications of bowel resection and anastomosis that can have devastating results in both humans and mice. A later complication observed in mice was small bowel obstruction, which again is a common late complication of any abdominal surgery due to the post-operative formation of adhesions. In terms of post-operative growth, the IL-10 KO mice were smaller then the WT mice at the start and throughout the experiments, which is consistent with the previously described 30% weight reduction in the majority of animals.[10] Both the sham and the ileocolonic anastomosis operations did not have a further influence on weight gain.

2.5. CONCLUSION

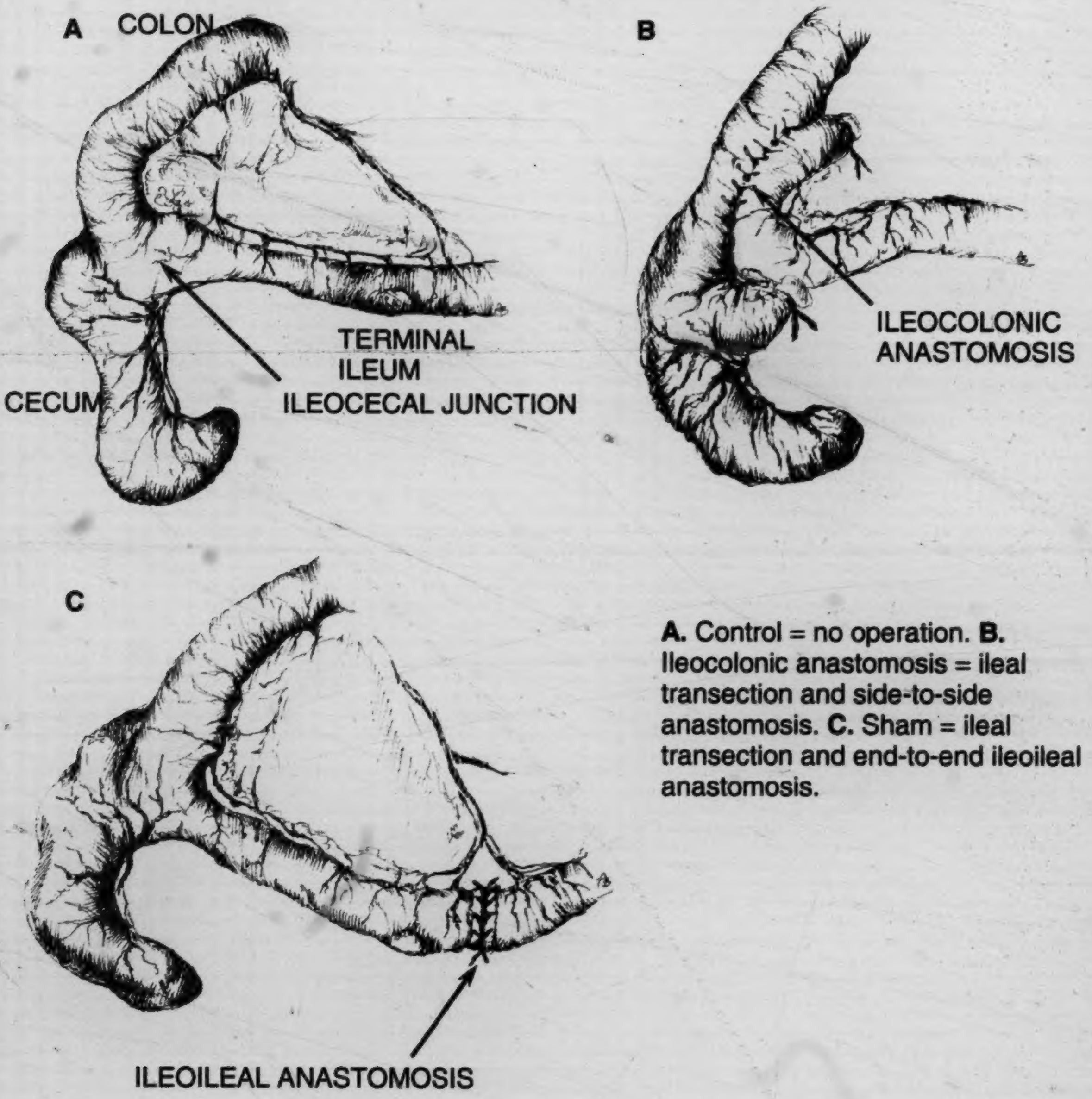
The surgical mouse model introduced in this chapter meets the requirements for a model of ileocolonic anastomosis in CD. Given the enormous number of unanswered questions regarding the post-operative course of CD, this model is likely to become a valuable tool for the characterization of post-operative pathophysiology that leads to disease recurrence. It may also prove an effective testing ground for potential therapeutics.

Table 2.1. Animal models of IBD

GENETICALLY ENGINEERED MODELS
Gene knockout models A20 knockout mouse IL-2 knockout/IL-2 Rα knockout mouse IL-10 knockout mouse IKK-γ (NEMO)/IKKαβ mouse Keratin $8^{-/-}$ knockout mouse MDR1 (Mdr1$^{-/-}$) knockout mouse STAT3 knockout mouse T-cell receptor mutant mouse TNF-3' UTR (TNF^{ARE}) knockout mouse Trefoil factor-deficient mouse
Transgenic models IL-7 transgenic mouse STAT4 transgenic mouse DN N-cadherin mouse HLA B27 transgenic rat
SPONTANEOUS COLITIS MODELS
SAMP/Yit mouse TNF-3' UTR/TNF ΔARE mouse A20 mouse
INDUCIBLE COLITIS MODELS
Trinitrobenzene sulfonic acid colitis Oxazolone colitis Dextran sulfate sodium colitis Carrageenan colitis Peptidoglycan-polysaccharide colitis
ADOPTIVE TRANSFER MODELS
CD4+/CD45RB high T-cell transfer colitis Hsp60-specific CD8+ T-cell transfer colitis

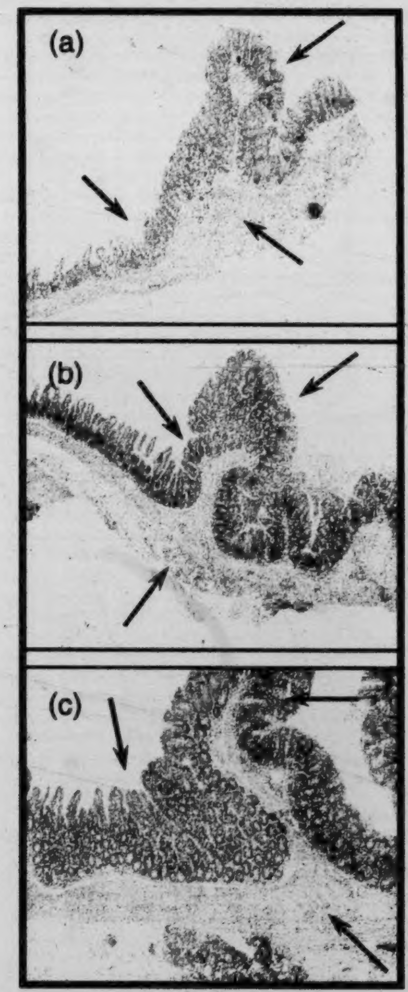
IL = interleukin; IKK = IκB kinase; R = receptor; MDR = multi-drug resistance; DN = dominant negative; TNF = tumor necrosis factor; UTR = untranslated region; Hsp = heat shock protein

Figure 2.1. Schematic representation of three surgical procedures


A
COLON
TERMINAL
ILEUM
CECUM
ILEOCECAL JUNCTION
B
ILEOCOLONIC
ANASTOMOSIS
C
ILEOILEAL ANASTOMOSIS

A. Control = no operation. **B.** Ileocolonic anastomosis = ileal transection and side-to-side anastomosis. **C.** Sham = ileal transection and end-to-end ileoileal anastomosis.

Figure 2.2. Histology of mouse ileocecal valve



Hematoxylin and eosin stain of the mouse ileocecal valve: (a) WT mouse 10X magnification, (b) IL-10 KO mouse 10X magnification and (c) IL-10 KO mouse 20X magnification. The ileocecal valve is characterized by a mucosal fold (red arrows), mucosal transition from villi in the terminal ileum to crypts (black arrows) I the colon, and thickening of the circular muscle (blue arrows).

Table 2.2. Mean length of the circular muscle thickening at the ileocecal junction in WT and IL-10 KO mice

Mouse	Mean length (cm)	SEM
WT F	0.62	0.09
WT M	0.57	0.12
WT F + M	0.59	0.07
IL-10 KO F	1.13	0.15
IL-10 KO M	1.06	0.17
IL-10 KO F + M	1.10	0.11
WT (F+M) + IL-10 KO (F+M)	1.00	0.09

The mean length of the circular muscle thickening at the ileocecal junction was significantly longer in the IL-10 KO mice (N = 12) than the WT (N = 12) mice ($p < 0.001$).

SEM = standard error of the mean; WT = wild type mice; IL-10 KO = IL-10 gene deficient mice; F = female; M = male.

Table 2.3. Mortality rates among WT and Il-10 KO mice

Mouse type	OR	N	Mortality Rate (%)
WT	C	19	0
	S	20	10
	E	22	23
Total		62	11
IL-10 KO	C	20	5
	S	21	10
	E	23	17
Total		64	11

There was no difference in mortality rates between the WT and IL-10 KO mice. WT = wild type mice; IL-10 KO = IL-10 gene deficient mice; OR = operation; C = control animals (no surgery); S = sham operation; E = experimental surgery (ileocolonic anastomosis); N = number of animals.

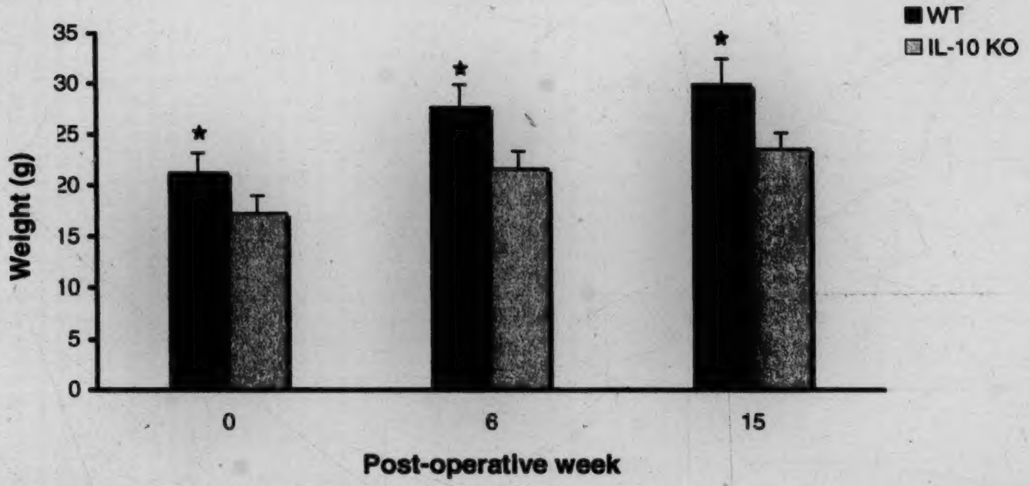
Table 2.4. Mortality event description

Mouse type	OR	POD#	Cause of death
WT	S	2	? Sepsis
WT	E	2	Leak at anastomosis
WT	E	3	Leak at anastomosis
WT	E	4	N/A
WT	E	17	N/A
WT	S	27	Obstruction at anastomosis
WT	E	49	N/A
IL-10 KO	E	4	N/A
IL-10 KO	E	5	Obstruction at anastomosis
IL-10 KO	C	7	Starved
IL-10 KO	E	7	Starved
IL-10 KO	E	24	No weight gain, colonic intussusception
IL-10 KO	S	43	SBO mid jejunum
IL-10 KO	S	65	? Aortic rupture

The most common causes of mortality (1 to 7 days after the operation) were anastomostic leak and obstruction at the anastomosis. Late causes of mortality (>7 days after surgery) were caused by events not related to anastomosis.

WT = wild type mice; IL-10 KO = IL-10 gene deficient mice; OR = operation; C = control animals (no surgery); S = sham operation; E = experimental surgery (ileocolonic anastomosis); POD# = post-operative day number.

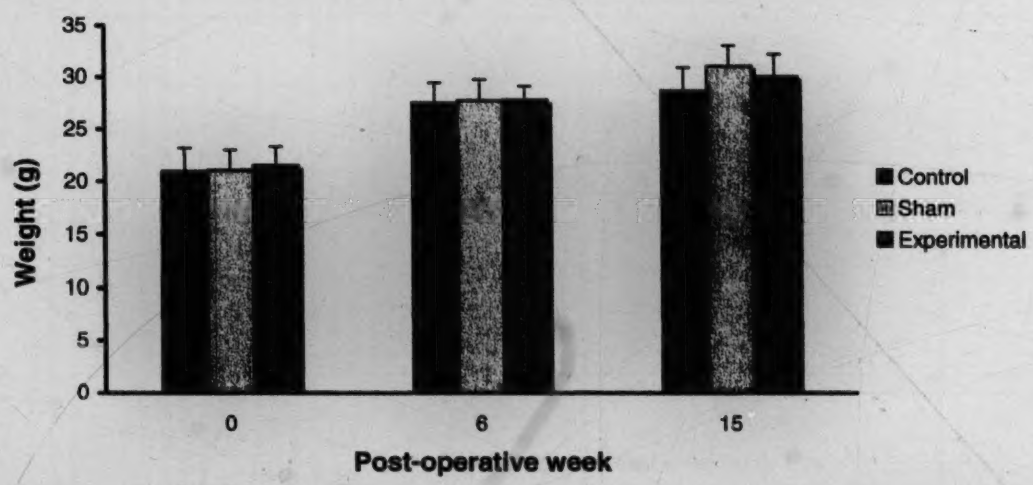
Figure 2.3. **Overall mean weight for WT and Il-10 KO mice at 6 and 15 weeks after surgery**



IL-10 KO mice (N = 36) we significantly smaller than the WT mice (N = 36) at the start of the study and at the 6-week and 15-week time points.

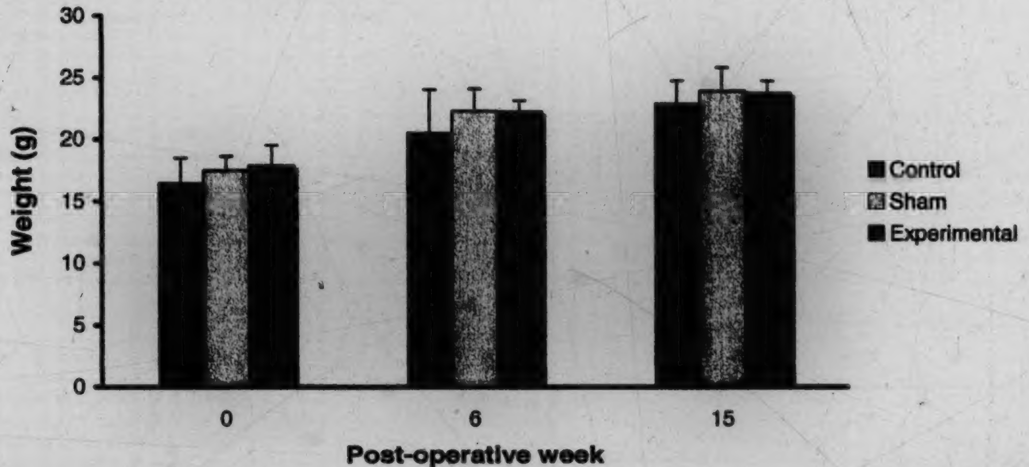
* signifies statistically significant difference between WT and IL-10 KO mice at each time point with $p < 0.001$.

Figure 2.4. Mean weight of WT mice according to type of surgery



In the WT mice, there was no statistical difference in the mean weight between the three surgical groups: control (N = 6), sham (N = 6), experimental = ileocolonic anastomosis (N = 6).

Figure 2.5. Mean weights of IL-10 KO mice according to type of surgery



In the IL-10 KO mice, there was no statistical difference in the mean weight between the three surgical groups: control (N = 6), sham (N = 6), experimental = ileocolonic anastomosis (N = 6).

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Chapter 3. Changes in Bacterial Profile of the Neo-terminal Ileum After Ileocolonic Anastomosis

3.1. INTRODUCTION

Normal Intestinal Bacterial Flora

The gastrointestinal tract is one of the most unique environments in the human body, where the properties of different compartments allow for growth of different numbers and populations of bacteria (Figure 3.1). For instance, the low pH of the stomach and rapid transit time allow very few bacteria to survive, yet just a few centimeters away in the duodenum, Gram-positive microbes such as *Streptococci* and *Lactobacilli* flourish in numbers of 10^3 organisms/mL of intestinal contents.[1] The number and diversity further increases towards the colon where the predominant populations of Gram-positive aerobes are progressively replaced by Gram-negatives and anaerobes reaching 10^8 bacteria/mL of contents in the ileum.[1] Probably the most drastic change in diversity, accompanied by a 100 to 1000 fold increase in numbers, is observed at the transition from the terminal ileum into the cecum/colon where the ileocecal valve provides a physical and functional barrier between the two compartments.[1] The major microbes residing in the colon are strict anaerobes and accurate enumeration has only recently become possible with the introduction of molecular techniques such as fluorescence in situ hybridization (FISH) and polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), both based on bacterial 16S rRNA.[2, 3] Based on culturing techniques the estimate of bacterial species in healthy human colons is 400 - 500 species, however, using molecular techniques this estimate markedly increases to 15,000 - 36,000 species.[1,4]

Although bacteria come into direct contact with an enormous surface area of epithelium armored with powerful immunological weapons, in healthy individuals these bacteria are tolerated and no immune response is generated. The recent explosion of research related to probiotics has provided evidence of the dynamic and often symbiotic relationship between commensal bacteria and the gastrointestinal epithelium. The functions of the intestinal flora that benefit the host can be divided into three categories: (1) protective functions such as pathogen displacement, competition for nutrients and production of anti-microbial factors, (2) structural functions, which include maintenance of apical barrier integrity, immune system development and induction of immunoglobulin A (IgA) secretion, and (3) metabolic functions.[5] Metabolic functions of intestinal microbes include vitamin synthesis, and breakdown of carcinogens and fermentation of non-digestible residues, such as starch, into short chain fatty acids, which serve as an additional energy source for humans. Other metabolic functions of intestinal flora include contribution to sodium and chloride absorption by the epithelium, and modulation of intestinal epithelial cell differentiation and proliferation.[5, 6] The combined genome of intestinal microbes surpasses the human genome in size, and the above functions are just a few examples of our rapidly expanding knowledge of the microbe-epithelium cross-talk that is essential for health. It is most likely that this cross-talk can explain many gastrointestinal diseases, while also offering potential therapeutic targets.[7]

Role of Microbes in Crohn's Disease and Post-operative Recurrence

Mycobacterium avium paratuberculosis was one of the first infectious agents implicated in the etiology of CD, however, to date this implication remains controversial.[8] In fact, no single infectious agent has been found to be responsible for disease onset or progression and none confers disease cure if removed. Nevertheless, as the roles of altered innate and adaptive immunity in CD are becoming more clearly defined, intestinal bacteria remain in the spotlight as one of the essential ingredients contributing to the observed intestinal pathology.

Current hypotheses of CD pathogenesis converge on the idea of multiple factors leading to disease. These factors include dysbiosis or imbalance between the protective and pathogenic bacteria, alterations in mucosal integrity such as permeability, and deregulated innate and adaptive immune responses.[9] With the introduction of more sensitive techniques for detection of bacterial diversity, many investigators have demonstrated that the composition of commensal bacteria as well as adherence patterns are altered in patients with IBD in comparison to individuals without bowel inflammation.[4] For instance, members of the phyla *Firmicutes* and *Bacteroides* have been shown to be depleted in patients with CD and ulcerative colitis (UC).[4] Furthermore, 16S rRNA analysis of ileocolonic biopsies from individuals with CD showed higher microbial variability between individuals and higher prevalence of *Clostridium* species and *E. coli* compared to biopsies from healthy individuals.[10] Mono-association studies, where the gastrointestinal tracts of animals under germ-free conditions are colonized with a single microorganism, have suggested that not all commensal bacterial species have the same pathogenic potential. For example, using the germ-free IL-10 KO model, *Escherichia coli* and *Enterococcus faecalis* have been shown to induce distinct patterns of bowel inflammation whereas *Pseudomonas fluorescens* does not cause disease at all.[11] Furthermore, certain bacteria can work in synergy and result in more severe bowel injury as is the case of dual-association of *Escherichia coli* and *Enterococcus faecalis* in germ-free IL-10 KO mice.[12] These examples are just a few of many that demonstrate the complexity of the microbial gastrointestinal ecosystem.

Studies have shown that commensal flora diversity is significantly diminished within inflamed tissues.[13] It is difficult to determine if the commensal flora alterations are the *result* or the *cause* of inflammation. Nevertheless, there is sufficient evidence to implicate commensal enteric flora in the pathogenesis of CD. A number of animal models of IBD under germ-free conditions, including the IL-10 KO mice, do not develop intestinal inflammation.[14] Germ-free IL-10 KO mice not only fail to develop colitis but there is also no evidence of immune system activation in these mice.[14] In humans and animal models, CD occurs in the segments of the gastrointestinal tract with the highest bacterial counts (terminal ileum, cecum and colon).[9] In patients with IBD (CD and UC) the number of mucosally adherent bacteria is increased and the microbial concentration is further elevated in lesions such as mucosal ulcers, fissures and abscesses.[15-17] These quantitative changes are present even in segments of bowel without obvious inflammation and become more drastic in areas of greatest disease severity.[18]

The commensal flora has also been implicated in the pathogenesis of CD recurrence after bowel resections involving ileocolonic anastomosis. A study by Neut *et al* [19] showed early colonization (within 3 months) of the neo-terminal ileum after ileocolonic anastomosis with increased numbers of bacteria and a composition that differed from the native terminal ileum population. These changes were absent in patients with ileostomy. In both, CD and non-CD (cancer) patients there was a dramatic increase in anaerobes but *E. coli* and enterococci were increased only in CD patients while *Bifidobacteria* and *Ruminococcus* counts were higher in non-CD patients. In this study, early recurrence was associated with higher counts of *E.coli* and bacteroides.[19]

Bacterial antigens contained in the fecal stream play a key role in post-operative recurrence of CD, as patients initially treated with a diverting ileostomy do not develop recurrent disease. However, the disease recurs as soon as gastrointestinal continuity is restored with closure of the ileostomy.[20] Similarly, infusion of luminal contents into excluded loops of bowel results in inflammatory changes within days of exposure to luminal contents, such as inflammatory cell recruitment.[21] Post-operative use of antibiotics (nitroimidazole family) delays symptomatic recurrence and decreases the severity of early recurrence. [22, 23] Unfortunately, the therapy is poorly tolerated by patients due to side effects of the antibiotics. The above evidence provides a convincing argument that commensal bacterial flora are not only involved in the pathogenesis of primary CD but also in the recurrence of CD after ileocolonic anastomosis.

In keeping with this evidence, the objective of our study was to investigate the bacterial changes in the neo-terminal ileum after ileocolonic anastomosis in IL-10 KO mice using a qualitative method of 16S rRNA amplification and DGGE. This molecular technique has revolutionized enumeration of microbes from various environments.[24] It is based on the amplification of a variable region of bacterial 16S ribosomal RNA using PCR and generation of equal length DNA fragments with unique nucleotide sequences representing different bacterial species.[25] A distinct bacterial "finger-print" from a given environment can then be visualized using a urea and formamide denaturing gradient acrylamide gel that generates a unique banding pattern based on the differences in the variable region DNA sequence. Each band then represents a different bacterial species.[25] Using PCR-DGGE, we were interested in assessing the changes in bacterial flora in the neo-terminal ileum after ileocolonic anastomosis. This assessment of bacterial changes was a pivotal step in testing our study hypothesis that commensal bacterial antigens drive disease recurrence through functional, immunological and histological changes (discussed in subsequent chapters).

3.2. MATERIALS AND METHODS

Animals and Surgeries

The animals and surgeries used were described in Chapter 2, in the Materials and Methods section.

DNA Isolation

At 6 weeks post-operatively, animals were euthanized by cervical dislocation. Segments of ascending colon, 2 cm in length, adjacent to the cecum and terminal ileum proximal to the ileocecal junction (in control and sham animals) or next to the ileocecal anastomosis (in experimental animals) were collected (Figure 3.2). The segments were cut in half longitudinally. One half of the segments including the bowel wall and contents were used for DNA isolation and the other half was used for histological analysis (Chapter 6). The collected tissues were placed in a sterile tube, snap frozen, and stored at -70ºC. On the day of DNA isolation, tissue was thawed in 1 mL of TN150 buffer (7 mM Tris-HCl, 4 mM Sigma 7-9, 148 mM NaCl, pH 8.0), vortexed and then centrifuged at 14,600*g* for 5 min at 4ºC. The pellet was washed twice in 1 mL of TN150 buffer and the tissue was disrupted with 300 mg of zirconium beads (0.1 mm diameter) at 2300*g* for 3 min. Next, the samples were centrifuged at 14,600*g* for 5 min and 500 μL of the supernatant containing the DNA underwent three sets of 500 μL buffer-saturated phenol / 500 μL chloroform-isoamyl alcohol (24:1) extractions. After the last wash, the DNA was precipitated overnight at -20ºC with 1 mL of 95% ethanol and 50 μL of 3 M sodium acetate. Following 20 min of centrifugation at 14,600*g*, the pellet containing DNA was dried and re-suspended in 30 μL of TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0). The DNA concentration in ng/μL and purity expressed as the absorbency ratio at 260/280 nm of 1.8 to 2.0 were determined using the Nanodrop 1000 [Thermo-Fisher Scientific, Fair Lawn, NJ]. The DNA samples were stored at -20ºC until further use.

DNA Based 16S rRNA Polymerase Chain Reaction

Intact genomic DNA following DNA isolation was confirmed on 1.5% agarose gel, using the Syber® Safe DNA gel stain [INVITROGEN, Carlsbad, CA], by the presence of a non-specific large molecular weight band near the wells. The 16S rRNA V3 region from the colonic and ileal samples was amplified in a GeneAmp PCR System 2400 [Applied Biosystems, Foster City, CA.] using the universal bacterial primers HDA1-GC and HDA2 (Figure 3.3) [GeNOsys, Provo, UT].[25, 26] Polymerase chain reaction (PCR) mixtures were set up containing: 5 μL of 10X PCR magnesium free PCR buffer, 1 μL of 10mM dNTP mixture, 1.5 μL of 50mM magnesium chloride, 0.2 μL of Platinum Taq® DNA polymerase [all reagents from INVITROGEN, Carlsbad, CA], 1 μL of 10 μM HDA1-GC and 1 μL of 10 μM HDA2 primers, 1 μL of DNA, and autoclaved distilled water to make up a total volume of 50 μL. The PCR consisted of 35 cycles of: 96ºC (4min), 94ºC (30 sec), 56ºC (30 sec), 72ºC (1 min) and 72ºC (4 min). The presence of the HDA PCR product was confirmed on 1.5% agarose gel as a 200 base pair band.

RNA Isolation

At 15 weeks post-operatively the animals were euthanized by cervical dislocation. The 2 cm segments of colon and terminal ileum were collected as described above with the exception that each segment was washed with 3 mL of phosphate buffered saline pH 7.2 [INVITROGEN, Carlsbad, CA], and divided into four pieces longitudinally. One of the pieces was homogenized with a sterile razor blade, and then snap frozen in liquid nitrogen while immersed in 500 μL of Trizol® Reagent

[INVITROGEN, Carlsbad, CA] and stored at -70ºC for RNA isolation at a later time. The RNA was isolated using RNeasy® Plus Mini kit [QIAGEN, Germantown, MD] following the manufacturer's instructions. In brief, the samples were thawed and an additional 500 µL of Trizol® Reagent was added. The samples were homogenized using plastic pestles and RNase free micro-centrifuge tubes [South Jersey Precision Tool & Mold Inc., Vineland, NJ] and then incubated at room temperature for 5 min. The samples were then centrifuged at 10,000*g* for 15 min at 4ºC and the supernatant was transferred to a gDNA spin column provided in the kit and centrifuged for 30 sec at 10,000*g*. The RNA in the flow through was precipitated with an equal volume of 70% ethanol and then allowed to bind to the RNeasy spin column by centrifuging for 15 sec at 10,000*g*. The RNA bound to spin column was washed three times, once with 700 µL of RW1 buffer and twice with 500 µL of RPE buffer. The RNA was eluted from the spin column with 30 µL of RNase-free water and centrifuged at 10,000*g* for 1 min. The RNA concentration in ng/µL and purity expressed as the absorbency ratio at 260/280 nm of 1.8 to 2.0 were determined using the Nanodrop 1000 [Thermo-Fisher Scientific, Fair Lawn, NJ.]. Aliquots of the RNA samples were used immediately for reverse transcription PCR (RT-PCR) and the remaining RNA was stored at -70ºC until further use.

RNA Based 16S rRNA Polymerase Chain Reaction

The 16S rRNA amplification from RNA samples was carried out as described for DNA with the exception of additional two steps: (1) confirmation that the RNA samples were not contaminated with DNA and (2) conversion of RNA to cDNA prior to amplification. To ensure the RNA samples were not contaminated with DNA, 16S rRNA amplification was carried out as described above except that instead of DNA substrate, 1 µL of RNA was added to the reaction tubes. Purity was confirmed by the absence of the

16S rRNA 200 base pair amplicon on 1.5% agarose gel. The cDNA was generated using a High Capacity cDNA Reverse Transcription Kit with RNase inhibitor [Applied Biosystems, Foster City, CA] as per the manufacturer's instructions. In brief, 60 µL reactions were set up containing the following reagents provided in the kit: 6 µL of 10RT buffer, 2.4 µL 25X dNTP mix, 6 µL of 10X RT random primers, 3 µL of MultiScribe™ reverse transcriptase, 3 µL of RNase inhibitor, and 9.6 µL of nuclease-free water. The PCR reagents were combined with 30 µL of 200 ng/µL RNA in RNase-free water. The RT-PCR was carried out using the GeneAmp PCR System 2400 [Applied Biosystems, Foster City, CA.] and consisted of one cycle of 25ºC (10 min), 37ºC (120 min) and 85ºC (5 sec). Subsequently, 16S rRNA was amplified and amplification was verified as described above with cDNA serving as a substrate instead of genomic DNA.

Denaturing Gradient Gel Electrophoresis

DGGE was performed using the Bio-Rad D-Code System [Hercules, CA] designed specifically for DGGE. Denaturing gel consisted of 6% acrylamide:bis acrylamide (37:1), 8 M urea, 20% deionized formamide, 1 x TAE (2 M Tris-HCl, 1M glacial acetic acid, 50 mM EDTA and distilled water), 0.1% ammonium persulfate and 0.05% TEMED. The gel was polymerized for 1 hour and stored overnight at 4ºC. The following day, the gel was assembled into the apparatus and 36 µL of sample was loaded into each well. Each 36 µL sample consisted of 6 µL pooled from six mice belonging to the same experimental group. The gel was run at 130V for 4 hours with a denaturing gradient of 22% to 55% urea/formamide. After the electrophoresis was completed, the gel was stained with ethidium bromide, and photographed under UV transillumination.

The similarity of the microbial structures was determined with comparison and clustering of the profiles generated from PCR-DGGE with the Bionumerics software package (Applied Maths, Austin, TX). Similarity matrices were produced using the Dice coefficient following the calculation:

$$C_s = \frac{2j}{(a + b)} \times 100$$

where *j* is the number of common bands in lane 1 and 2, *a* is the number of PCR-DGGE bands in lane 1, and *b* is the number of PCR-DGGE bands in lane 2.[27] Two identical profiles generate a C_s value of 100% and two completely different profiles generate a C_s value of 0%.[27] The gel at 6 weeks and similarity calculations were conducted in duplicate to ensure the results were reproducible.

Statistical Analysis

Since analysis was carried out based on groups of animals by pooling DNA or RNA from individual mice into respective groups, the calculations of mean, variance and statistical significant were not applicable.

3.3. RESULTS

Similarity Between the Colon and Ileum at 6 Weeks Post-op (Figure 3.4A)

At the 6-week time point, similarity coefficient (C_s) was significantly higher in both the WT and IL-10 KO mice that underwent ileocolonic anastomosis than the control animals (no surgery). In WT control mice the similarity between the colon and ileum was 52.9% and increased to 82.4% following ileocolonic anastomosis (Table 3.1). In the sham-operated animals the similarity was 74.6%. This was comparable to the IL-10 KO mice where prior to surgery there was 58.2% bacterial similarity between the colon and ileum and 85.4% similarity after ileocolonic anastomosis. The sham-operated animals had 45.5% similarity.

Similarity Between the Colon and Ileum at 15 Weeks Post-op (Figure 3.4B)

At 15 weeks after surgery, the bacterial profiles in the ileum and colon were analyzed using RNA based PCR-DGGE. In contrast to the results at 6 weeks, which were based on DNA, there was a higher baseline similarity between the colon and ileum and a smaller change in the similarity coefficient after ileocolonic anastomosis. Eighty-four percent and 81% similarities were observed in the WT control and sham-operated animals, respectively. Animals that underwent ileocolonic anastomosis had a 90% similarity in the bacterial population between the colon and ileum (Table 3.1). Likewise, the IL-10 KO control mice had 81% similarity between the colon and ileum while the similarity in the sham group was 71%. The similarity increased to 85% in the animals that underwent ileocolonic anastomosis.

3.4. DISCUSSION

In our study, after ileocolonic anastomosis there was a notable early (at 6 weeks) change in the bacterial composition in the neo-terminal ileum in both the WT and IL-10 KO mice. The bacterial populations in the neo-terminal ileum and colon were more similar suggesting mixing of the ileal and colonic populations after the elimination of the ileocecal valve. We speculate that the observed increase in similarity is due to a reflux, or accelerated growth of colonic bacteria into the small intestine and migration of ileal species into the colon. Our finding of changed microbial flora in the neo-terminal ileum is consistent with a previous report that described early colonization by colonic flora of the neo-terminal ileum after ileocolonic anastomosis in both CD and non-CD patients. [19] In this study by Neut *et al*, both sets of patients had a dramatic increase in the anaerobic populations detected 3 months after surgery and still present at 1 year after the procedure.[19] Furthermore, an increased number of *Escherchia coli* and enterococci were present in CD patients.[19]

We also observed an unexpectedly high ileum to colon similarity in the WT mice following the sham operation. A plausible explanation for this finding is related to the surgery itself. In a number of animals undergoing this operation, the transection of the terminal ileum and end-to-end anastomosis was carried out in close proximity to the ileocecal junction (within 1 cm). This technical approach, with its close proximity to the ileocecal valve may have led to at least a partial disruption of the ileocecal valve mechanism allowing for some colonic reflux into the small bowel and increased colonization of the terminal ileum with colonic microbes.

We did not undertake identification of bacterial species present in our samples. PCR-DGGE is a qualitative study of bacterial diversity that allows for detection of similarity and differences in the bacterial composition from diverse environments or in response to a given treatment or disease state. It can also identify the presence of specific species through sequencing of bands present on the DGGE gel. Our hypothesis and study goals were to identify any immune and functional changes, as well as mucosal injury after ileocolonic anastomosis driven by the changes in the microbial flora in the neo-terminal ileum. In the experiments outlined in this Chapter we sought to demonstrate that ileocolonic anastomosis leads to alterations in the bacterial populations in the neo-terminal ileum as related to colon before and after ileocolonic anastomosis. Additional experiments are needed to correlate this colonization with immune, functional and histological changes. Subsequently it will be important to identify exact bacterial species present, because previous studies have shown that not all gastrointestinal bacteria inflict the same inflammatory effect on the bowel.[11]

The comparison of the colon to ileum at 15 weeks post-operatively yielded dissimilar results relative to the 6-week time point. At 15 weeks, in both WT and IL-10 KO mice, there was a higher baseline similarity between the colon and ileum in control (no surgery) and sham-operated animals. The similarity increased minimally after ileocolonic anastomosis; 7.2% in the WT and 4.8% in the IL-10 KO mice. In contrast, at 6 weeks after surgery, the similarity increased by 55.8% in the WT and 46.8% in the IL-10 KO mice after ileocolonic anastomosis. This difference may have several explanations. First, the natural process of aging may lead to a different colonization profile in the ileum. Alternatively, the observed difference between the two time points may stem from the different type of nucleic acid used for the PCR-DGGE analysis. DNA was used for the 6-week time point and RNA for the 15-week time point analysis. We changed to RNA at 15 weeks as we were interested in examining only bacteria adherent to bowel wall and we speculated RNA would be a more sensitive method. The discrepancy in the DGGE bands between the same samples based on DNA and RNA have been described previously and result from the fact that the two methods measure distinct bacterial population characteristics.[28] The copy number of rRNA gene operons per chromosome is more constant between bacteria and the DNA-based DGGE is more accurate at detecting diversity regardless of viability and typically this profile has more bands.[28, 29] The RNA-based PCR-DGGE represents bacterial ribosomal content, which can be extrapolated to correspond to bacteria that are most metabolically active, with the fastest growth rates and substantial biomass.[28]

This suggests that at the 6-week time point we may have measured bacterial diversity and showed that the bacterial diversity became more alike between the neo-terminal ileum and colon after ileocolonic anastomosis. At 15 weeks, however, we most likely measured the dominant bacterial flora in terms of metabolic activity and biomass and showed that when looking at these population parameters (metabolic activity and biomass) perhaps the terminal ileum and colon are highly similar at baseline (without ileocolonic anastomosis). The terminal ileum has intrinsically higher bacterial counts than the proximal gastrointestinal tract (stomach, duodenum and jejunum) and this is in part due to some colonic content reflux into small bowel even in the presence of a functional ileocecal valve and stasis at the ileocecal valve allowing for bacterial overgrowth.[1] Perhaps the dominant bacteria are very similar on both sides of the ileocecal valve, suggesting that perhaps specific species rather than most metabolically active or abundant bacteria play a role in disease recurrence. For instance, previously mentioned mono-association studies in germ-free animals have demonstrated that not all bacteria can illicit an immune response.[11] In addition, in the study by Neut *et al,* ileocolonic anastomosis led to an equivalent colonization of the neo-terminal ileum with *Bacteroides* in both the CD and non-CD patients, yet only in the CD patients was *Bacteroides* associated with increased endoscopic recurrence at three months and one year after surgery.[19] Perhaps 'who' is present is one of the factors contributing to disease recurrence in the neo-terminal ileum but "how many" are present may also be significant. The latter was not truly addressed by our study, as although the RNA based PCR-DGGE provided some information on the metabolically active bacteria, we did not quantify the bacteria in the neo-terminal ileum before and after ileocolonic anastomosis. As a result at this time we cannot draw definitive conclusions based on our experiments if the number of bacteria in the neo-terminal ileum increase and if so, what impact this change has on disease recurrence.

3.5. CONCLUSION

We demonstrated that ileocolonic anastomosis in the WT and IL-10 KO mouse leads to increased similarity in the bacterial populations between the ileum and colon at 6 weeks after surgery. At 15 weeks after ileocolonic anastomosis we showed minimal changes in the profile of the most metabolically active microbes. Ileocolonic anastomosis alters bacterial diversity in the small bowel but may not change the dominant populations suggesting that it may be the specific bacterial species rather than the most abundant bacteria that drive the inflammatory changes leading to post-operative disease recurrence. The changes in quantity of bacteria may also play a role in disease recurrence but this was not addressed by our study at this time. It is difficult to compare the two time points in our study as different nucleic acids were used for the PCR-DGGE analysis (DNA was used at 6 weeks and RNA at 15 weeks). Future studies looking at RNA based PCR-DGGE at 6 weeks and DNA based PCR-DGGE at 15 weeks along with identification of species and quantification of bacteria present in the neo-terminal ileum following ileocolonic anastomosis would provide valuable information for more definitive conclusions.

Figure 3.1. Distribution of commensal bacteria in human gastrointestinal tract

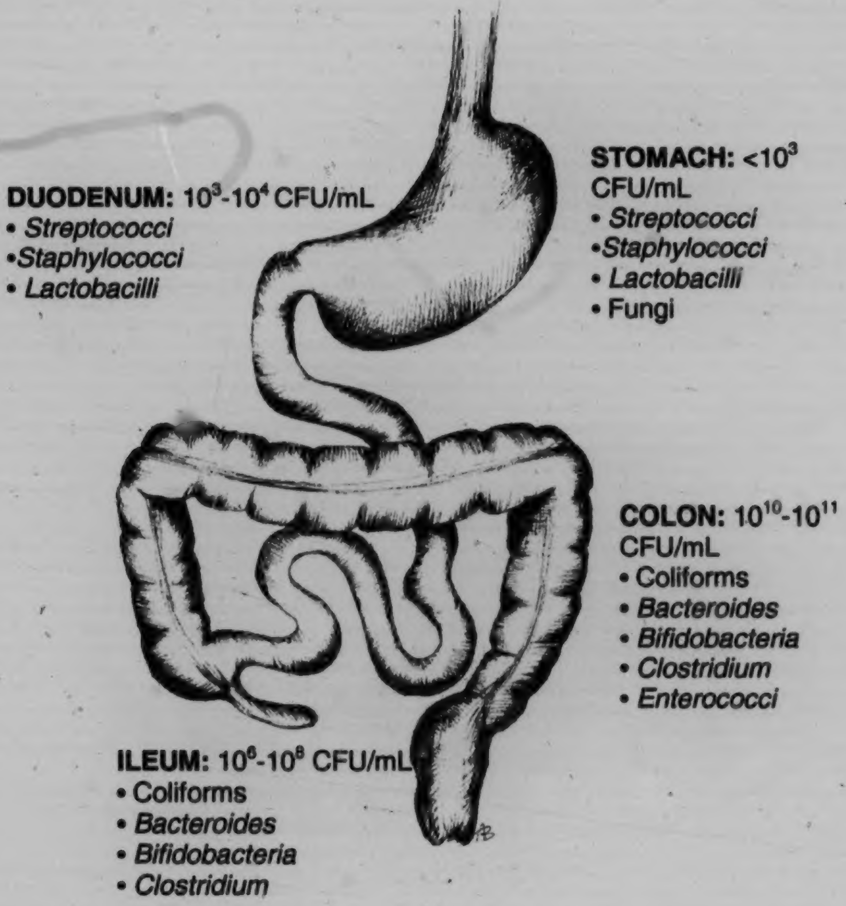
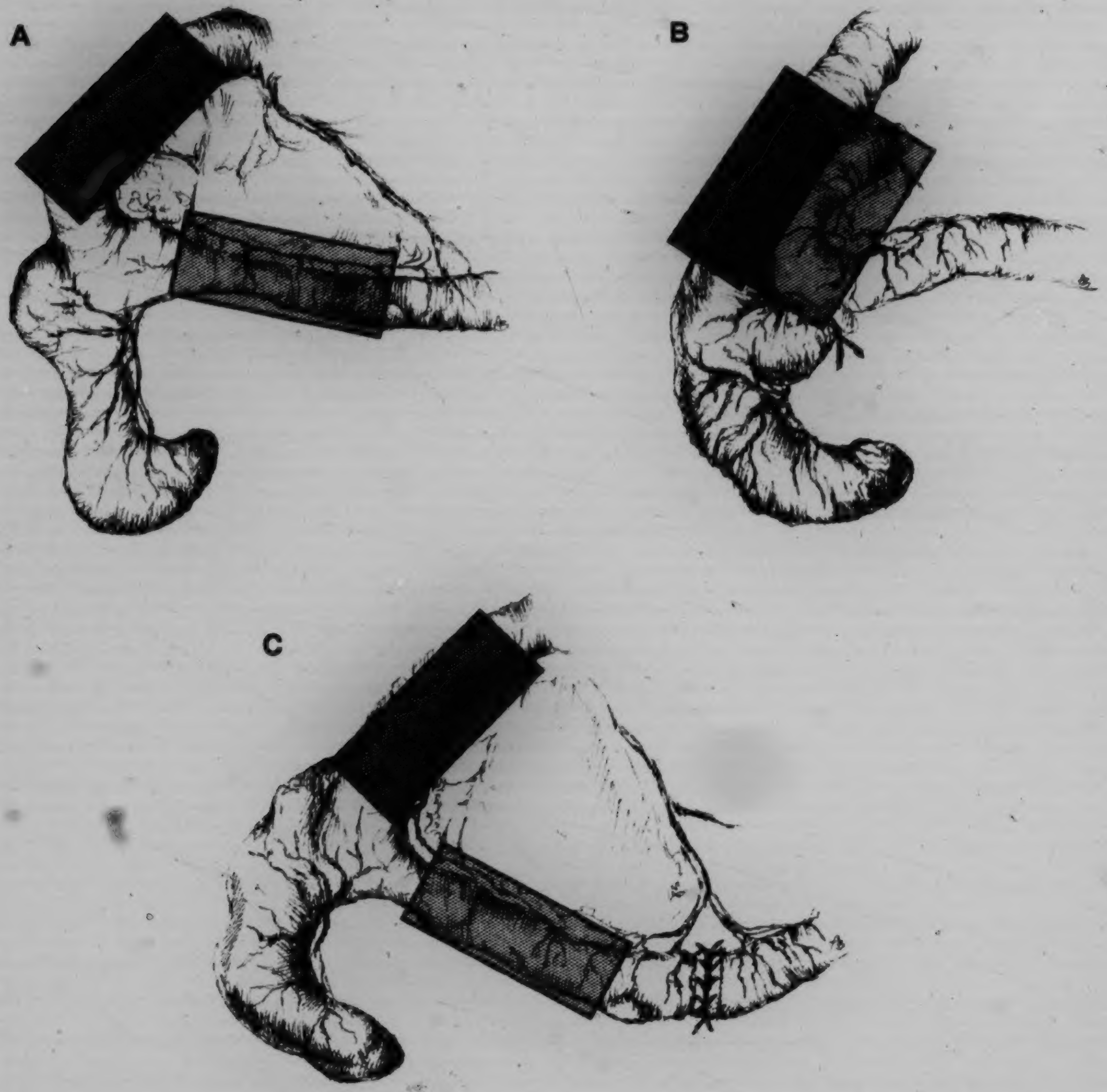

STOMACH: $<10^3$ CFU/mL
• *Streptococci*
•*Staphylococci*
• *Lactobacilli*
• Fungi
DUODENUM: 10^3-10^4 CFU/mL
• *Streptococci*
•*Staphylococci*
• *Lactobacilli*
COLON: 10^{10}-10^{11} CFU/mL
• Coliforms
• *Bacteroides*
• *Bifidobacteria*
• *Clostridium*
• *Enterococci*
ILEUM: 10^6-10^8 CFU/mL
• Coliforms
• *Bacteroides*
• *Bifidobacteria*
• *Clostridium*

Figure 3.2. Bowel segments collected for 16S rRNA PCR-DGGE analysis



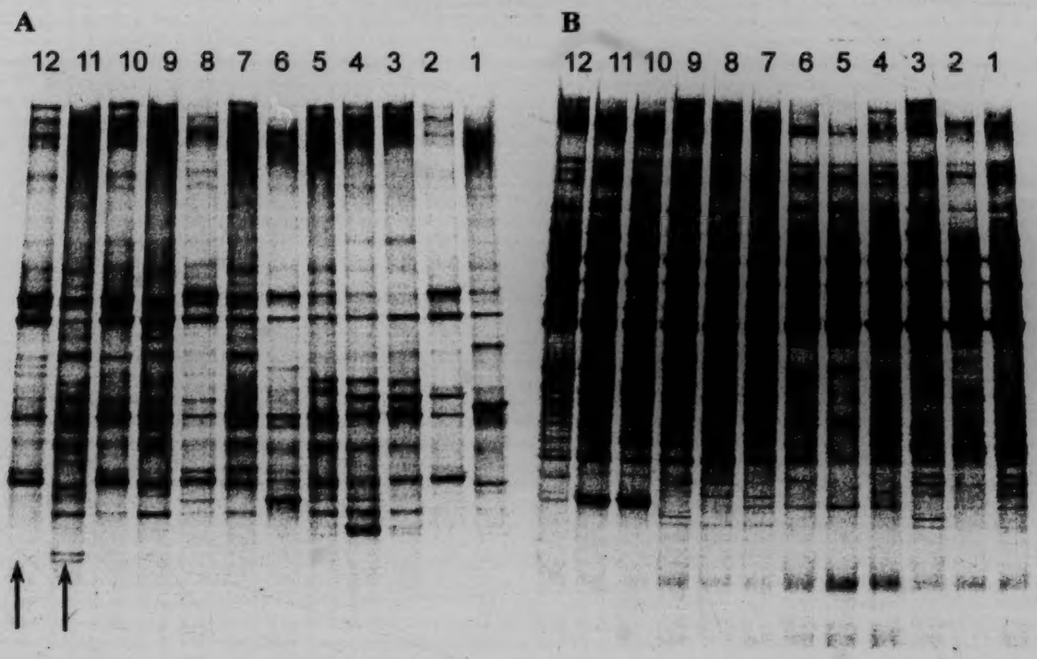
A. Control. **B.** Ileocolonic anastomosis. **C.** Sham operation. Red (colon) and green (terminal ileum/neo-terminal ileum) boxes denote 2 cm segments of collected bowel.

Figure 3.3. Sequences for HDA1-GC/HDA2 primers used in 16S rRNA PCR-DGGE

HDA1-GC*	**5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG-3'
HDA2	5'-GTA TTA CCG CGG CTG CTG GCAC-3'

* In boldface is the GC clamp.

Figure 3.4. 16S rRNA PCR-DGGE gel of colonic and ileal bacteria at 6 weeks and 15 weeks after surgery



A. DNA based DGGE at 6 weeks after surgery. **B.** RNA based DGGE at 15 weeks after surgery. Each lane (marked with an arrow) represents nucleic acid from a group of animals undergoing the same procedure.

Lane legend:
1 = WT control colon
2 = WT control ileum
3 = WT ileocolonic anastomosis colon
4 = WT ileocolonic anastomosis ileum
5 = WT sham colon
6 = WT sham ileum

7 = IL-10 KO control colon
8 = IL-10 KO control ileum
9 = IL-10 KO ileocolonic anastomosis colon
10 = IL-10 KO ileocolonic anastomosis ileum
11 = IL-10 KO sham colon
12 = IL-10 KO sham ileum

Table 3.1. Percentage similarity between colon and ileum for WT and IL-10 KO mice before and after ileocolonic anastomosis at 6 and 15 weeks after the operation

Mouse	Operation	Similarity (%) at 6 weeks based on DNA	Similarity (%) at 15 weeks based on RNA
WT	*Control*	52.9	84.9
	Sham	74.6	81.2
	Ileocolonic anastomosis	82.4	89.8
IL-10 KO	*Control*	58.1	81.5
	Sham	45.5	70.9
	Ileocolonic anstomosis	85.4	85.4

Based on bacterial DNA, at 6 weeks after ileocolonic anastomosis the similarity between the colon and ileum increased in both the WT and IL-10 KO mice. Based on bacterial RNA, at 15 weeks after ileocolonic anastomosis the similarity between the colon and ileum increased minimally.

WT = wild-type mice, IL-10 KO = IL-10 gene deficient mice.

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Chapter 4. Functional Small Bowel Changes After Ileocolonic Anastomosis - Glucose Transport

4.1. INTRODUCTION

Gastrointestinal Glucose Transport

In humans, carbohydrates make up approximately 50% of the daily caloric intake.[1] Glucose is the basic unit of carbohydrate metabolism and its absorption takes place in the top one third of the villi in the small intestine.[2] In the early 1950's glucose absorption was described to be an 'active' process.[3] Today, glucose absorption is understood to be in part a Na^+ coupled, energy-dependent process. SGLT1 is a cell membrane co-transporter located in the brush border of the enterocytes.[4] It uses Na^+ electrochemical potential gradient to co-transport two molecules of Na^+ and one molecule of glucose across the luminal membrane (Figure 4.1).[2, 5] The process is energy-dependent since the maintenance of Na^+ gradient requires Na^+/K^+-ATPase pump, which uses energy to co-transport Na^+ out of the cell across the basolateral membrane (Figure 4.1).[2] Once inside the enterocyte, majority of glucose is transported across the basolateral membrane into the portal circulation via facilitative diffusion (down its concentration gradient) through another transporter, GLUT2 (Figure 4.1).[2, 6] For a long time the existence of a second brush border transporter of glucose based on facilitative diffusion has been speculated but GLUT2 was thought to be confined to the basolateral side.[7, 8] The solution to this dilemma came from recent research demonstrating insertion of GLUT2 into brush border membrane and diffusive absorption of glucose in response to high luminal concentration of glucose, glucagon-like peptide 2, activation of AMP-activated protein kinase and even psychological stress.[9-13] AMP-activated protein kinase serves as a cellular 'gate-keeper' limiting select ATP-dependent processes such as the SGLT1 glucose transport during cellular stress and energy depletion.[12] Psychological stress in rats induced by water avoidance, has been shown to decrease Na^+-dependent glucose transport and increase the brush-border GLUT2 mediated glucose uptake.[13]

Given that stress seems to cause major disturbances in the intestinal glucose handling, our objective was to determine the effect surgical stress and changes in the intestinal, bacterial colonization would have on intestinal glucose transport. We were interested in assessing the functionality of the neo-terminal ileum after ileocolonic anastomosis and using glucose transport as a surrogate marker of that functionality. We hypothesized that surgical stress and increased colonization of the neo-terminal ileum after ileocolonic anastomosis causing inflammatory changes, would lead to decrease in sodium-dependent glucose transport that would fall on the continuum of global disturbances present in the post-operative period. To address our objective we used Ussing chambers to determine the net mucosal-to-serosal flux of tritium-labeled 3-*O*-methylglucose (3-OMG) in segments of terminal ileum from IL-10 KO mice 6 weeks after ileocolonic anastomosis.

4.2. MATERIALS AND METHODS

Animals and Surgeries

The animals and surgeries used were the same as previously described, for details please refer to Chapter 2, under the Materials and Methods section.

Glucose Transport Measurements

Mice were euthanized by cervical dislocation at 6 weeks after surgery and terminal ileum segments were mounted in Ussing chambers in quadruplicates [World Precision Instruments, Narco Scientific, Mississauga, ON] with exposed area of 0.196 cm^2. The segments were bathed on each side with 10 mL of oxygenated Normal Ringer's solution [in mmol/L: 1.1 $MgCl_2$, 1.25 $CaCl_2$, 114.0 NaCl, 5.0 KCl, 25.0 $NaHCO_3$, 1.6 Na_2HPO_4, 0.3 NaH_2PO_4] containing 20mM of 3-*O*-Methyl-D-glucose [SIGMA, St. Louis, MO]. 10 mmol/L of fructose was added to both mucosal and serosal sides and temperature was maintained at 37°C with water jacket and circulated by CO_2/O_2. The spontaneous transmural potential difference (PD) was measured with electrodes connected to the chambers with KCl-Agar (3M) bridges. The tissues were continuously short-circuited (I_{sc}) with an automatic voltage clamp [DVC 1000 World Precision Instruments, New Haven, CT] with the exception of 5 to 10 sec periods every 5 min when the PD across the mucosa was measured. Conductance (G) was calculated using PD measurements and Ohm's law.

For glucose fluxes, 5 μCi of non-metabolizable glucose, 3-*O*-Methyl-D-[1-^{3}H]-glucose [GE Healthcare, Waukesha, WI] was added either to mucosal or serosal side of the mounted tissues. After 20 min of equilibration, and four sets of flux measurements the viability of the tissue was assured by measuring I_{sc} 5 min after the addition of 10 μL of 10 mg/mL Forskolin [Calbiochem, La Jolla, CA], an activator of adenylate cyclase and observing an increase. Net directional flux from mucosal-to-serosal side (J_{net}) was calculated for conductance-matched tissues based on the following equation:

$$J_{net} = J_{mucosal} - J_{serosal}$$

where $J_{mucosal}$ is the measured glucose flux from mucosal-to-serosal side and $J_{serosal}$ is the measured glucose flux from serosal-to-mucosal slide.

The glucose fluxes were expressed in nmol/cm^2/h.

Statistical Analysis

Statistical analysis was performed using Stata 10.0 [Statacorp LP, College Station, TX]. Figures were generated using Excel Microsoft 2004 [Redmond, WA]. The glucose fluxes were expressed as means ± SEM. The statistical analysis of each measurement was performed using Kruskal-Wallis test. If the null hypothesis of the Kruskal-Wallis test was rejected, a multiple pair-wise comparison was performed using Mann-Whitney U test and Bonferroni's correction was applied. We considered $p < 0.05$ to be statistically significant unless multiple group comparison was conducted, in which case significant p-value was less than 0.05 divided by the number of groups being compared.

4.3. RESULTS

Glucose Transport

Mucosa-to-serosa (J_{mucosa})

We observed a statistically significant difference between the WT and IL-10 KO mice in the level of glucose transport from the mucosal-to-serosal side ($p < 0.001$). In the WT mice, glucose flux was higher than in the IL-10 KO mice; 3[illegible]10.83 ± 510.72

nmol/cm^2/h for WT control, 3366.05 ± 648.94 nmol/cm^2/h for WT sham and 3781.07 ± 309.99 nmol/cm^2/h for WT ileocolonic anastomosis (Figure 4.2). There was no statistical difference between these three WT groups ($p = 0.756$). The mucosal-to-serosal glucose flux was lower in the IL-10 KO mice. IL-10 KO control animals had a flux of 1716.79 ± 242.38 nmol/cm^2/h, while the sham and experimental groups had glucose fluxes of 1884.10 ± 247.60 nmol/cm^2/h and 1443.32 ± 199.62 nmol/cm^2/h, respectively (Figure 4.2). In the IL-10 KO mice there was also no statistically significant difference in the three surgical groups ($p = 0.543$).

Serosa-to-mucosa (J_{serosa})

We observed a trend towards increasing serosal-to-mucosal glucose flux among the WT mice that underwent either the sham or the ileocolonic anastomosis operation. This flux was 2514.26 ± 454.23 nmol/cm^2/h in WT controls and increased to 2962.08 ± 334.57 nmol/cm^2/h and 3216.98 ± 1033.78 nmol/cm^2/h in the sham and ileocolonic anastomosis groups respectively (Figure 4.3). There was no statistically significant difference between the three groups ($p = 0.642$). The serosal-to-mucosal flux was statistically lower in the IL-10 KO (control: 2095.99 ± 438.17 nmol/cm^2/h, sham: 2037.89 ± 444.36 nmol/cm^2/h and ileocolonic anastomosis: 1790.99 ± 215.97 nmol/cm^2/h) compared to the WT mice ($p = 0.050$) (Figure 4.3). There was no significant difference between the IL-10 KO mice in the three surgical groups ($p = 0.863$).

Net mucosa-to-serosa (J_{net})

The net mucosal-to-serosal glucose flux was calculated as described in the methodology section. In the WT mice there was a net reduction in glucose transport after both surgeries, sham (403.97 nmol/cm^2/h) and ileocolonic anastomosis (564.09 nmol/cm^2/h) relative to the control animals (1291.57 nmol/cm^2/h), however, this reduction did not reach statistical significance ($p = 0.109$) (Figure 4.4). There was also a trend towards lower glucose transport in the IL-10 KO mice relative to the WT mice, which did not reach statistical significance ($p = 0.053$). IL-10 KO control group had a net flux of -379.20 nmol/cm^2/h, sham group -153.79 nmol/cm^2/h and ileocolonic anastomosis group -347.67 nmol/cm^2/h. There was no statistical difference between the groups ($p = 0.056$) (Figure 4.4).

4.4. DISCUSSION

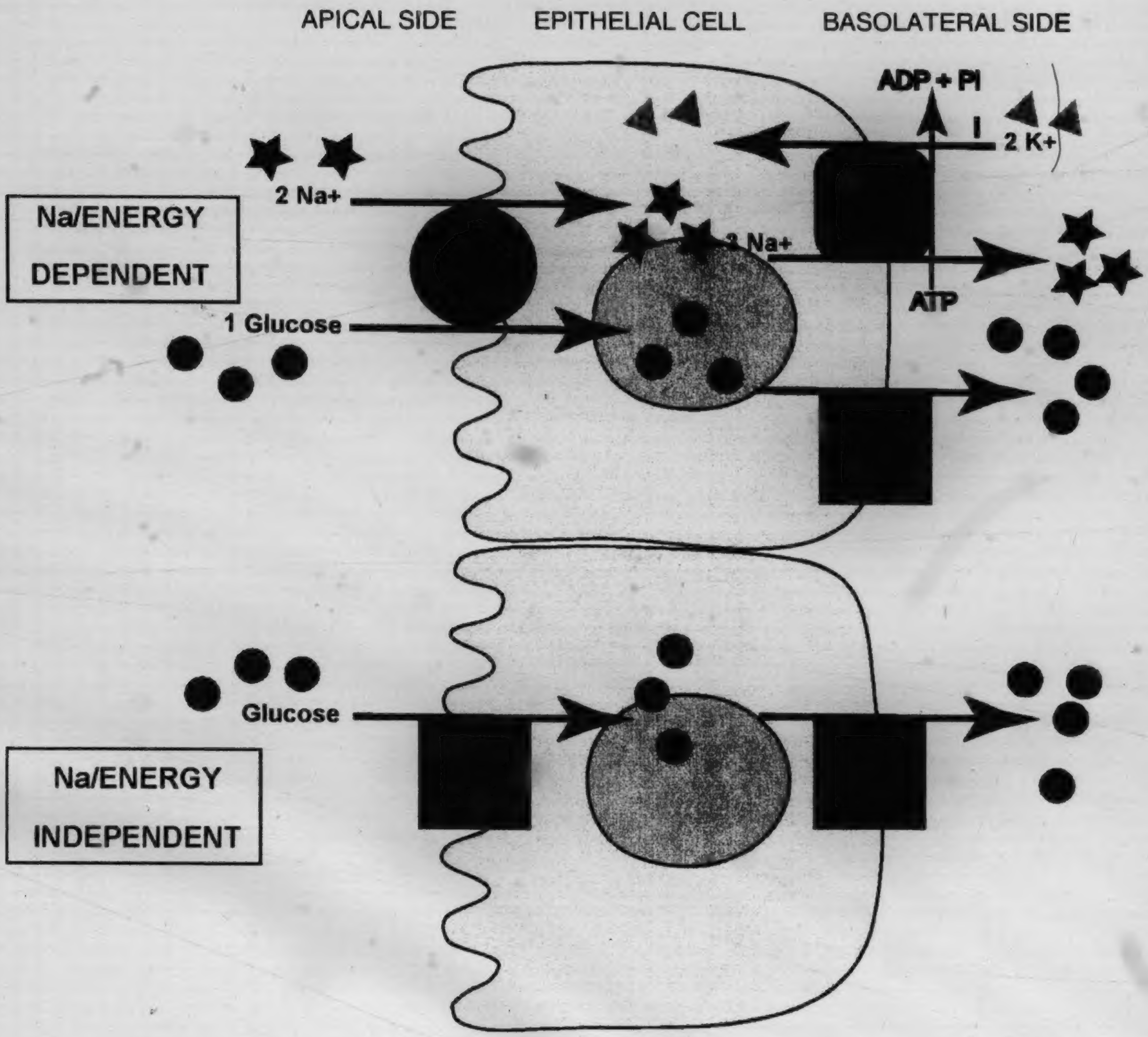
In our study we have demonstrated a significant difference in the glucose flux between the WT and IL-10 KO mice. The IL-10 KO mice had reduced mucosal-to-serosal, and serosal-to mucosal glucose fluxes as well as a trend towards decrease in the net glucose transport, compared to WT. There are a number of mechanisms that offer plausible explanations for the observed differences in glucose transport including architectural changes of the villi leading to an alteration in the enterocyte maturation and decreased absorptive surface area.[14, 15] Small bowel is composed of mucosal crypts and finger-like projections; the villi. The small bowel undergoes constant epithelial cell turnover where stem cells in the crypts give rise to new enterocytes that mature as they migrate towards the villi tips where they perform specialized functions such as glucose transport and subsequently undergo apoptosis.[14] As previously mentioned, glucose absorption is performed by mature enterocytes in the top one third of the villi.[2] Abnormal architecture of the mucosa in the IL-10 KO mice has been previously described.[15] Kuhn *et al* showed severe disturbances in the organization of the villi and crypts within the small bowel of IL-10 KO mice such as branching, fusions and regions of complete villi loss.[15] These architectural changes can decrease both enterocyte maturation and surface area for nutrient absorption, resulting in the observed reduced glucose transport in IL-10 KO mice. Since, these severe changes were described in IL-10 KO mice that routinely developed inflammation of the entire small bowel, which is absent in our IL-10 KO colonies, the architectural mucosal changes may only partially explain the findings in our study. Perhaps there is a differential expression of SGLT1 and GLUT2 in the WT and IL-10 KO mice. Despite lacking small bowel inflammation the IL-10 KO mice in our colonies still develop severe colonic inflammation, which can be considered a global state of stress and various types of stress have been shown to change the apical membrane expression of SGLT1 and GLUT2 as previously described.[12, 13]Further experiments measuring the expression of SGLT1 and GLUT2 are required to fully understand the mechanism behind the observed differences between the WT and IL-10 KO animals.

The aim of our study was to determine if surgical manipulation namely ileocolonic anastomosis would result in glucose transport disturbances. In the WT mice we observed two trends of increasing serosal-to-mucosal flux and decreasing net glucose transport that were present in sham and ileocolonic anastomosis groups. In the IL-10 KO mice there was no statistically significant difference in glucose fluxes (mucosal-to-serosal and serosal-to-mucosal) but there was a trend towards reduced net glucose transport in the surgical groups (sham and ileocolonic anastomosis). There are a number of likely explanations for our findings. First, the observed trends in the WT and IL-10 KO mice that were not statistically significant can be due to high variability between individual animals. This variability can be in part explained by relatively small number of animals in each group (N=6) and the fact that consistency in measurements using Ussing chambers requires complete mastery of the technique and our data was collected during a learning curve. Second, perhaps at 6 weeks after surgery the bowel has nearly completely recovered and the observed trends reflect the tail end of earlier glucose transport disturbances. Future studies looking at an earlier post-operative time course of glucose transport can truly answer the questions of post-operative glucose transport. Thirdly, it is possible the small bowel of IL-10 KO mice intrinsically has low levels of glucose transport and we were unable to detect any changes following surgery. As mentioned earlier, future examination of SGLT1 and GLUT2 expression can potentially offer some clarification in this area.

4.5. CONCLUSION

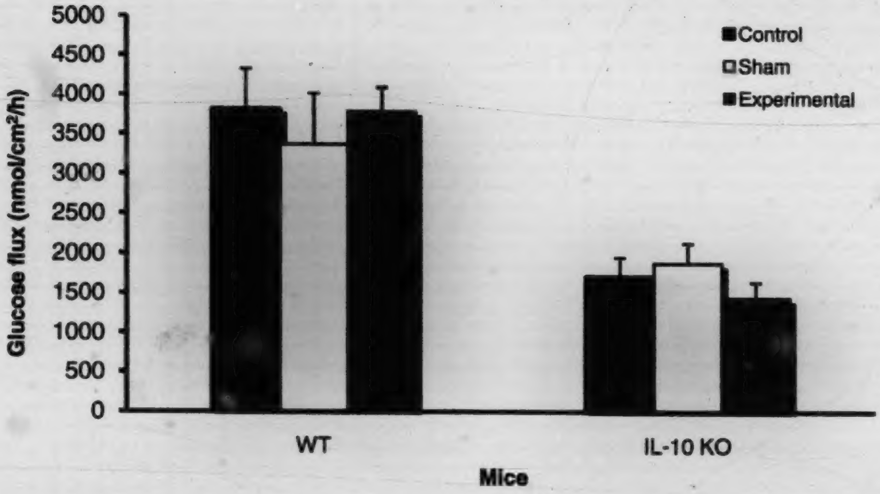
Overall, we showed there is a significant difference in the ileum's ability to handle glucose between the WT and IL-10 KO mice; in IL-10 KO mice glucose transport was reduced. There was no statistically significant difference in glucose transport between the three groups of surgeries (control, sham, ileocolonic anastomosis) among the WT and IL-10 KO mice. There was, however, a tendency towards decreased glucose transport with any surgical manipulation (sham and ileocolonic anastomosis). Since changes were present in the sham-operated and ileocolonic anastomosis animals, this suggests that surgery alone might be able influence glucose transport in the small bowel and that increased bacterial colonization of the small bowel may not play a role. Future studies with larger number of animals, an earlier post-operative time course and characterization of glucose transporters expression might be able to clarify our findings.

Figure 4.1. Glucose transport by intestinal epithelium


APICAL SIDE
EPITHELIAL CELL
BASOLATERAL SIDE
ADP + PI
2 K+
2 Na+
Na/ENERGY DEPENDENT
3 Na+
ATP
1 Glucose
Glucose
Na/ENERGY INDEPENDENT

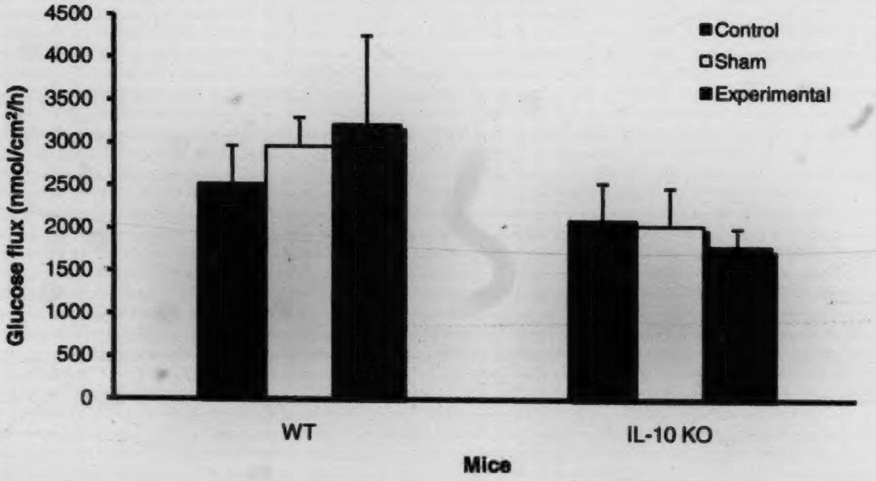
Two mechanism of glucose transport by intestinal epithelium: (1) via SGLT1 trasporter (top of figure) and (2) via GLUT2 transporter (bottom of figure). SGLT1 is a Na^+ and energy depenedent process as Na+ gradient is required, which is generated by Na^+/K^+ ATPase pump on the basolateral side. GLUT2 is Na^+ and energy indepenedent and is referred to as diffussive process.

Figure 4.2. Mucosal-to-serosal ($J_{mucosal}$) glucose flux in WT and IL-10 KO mice at 6 weeks after surgery



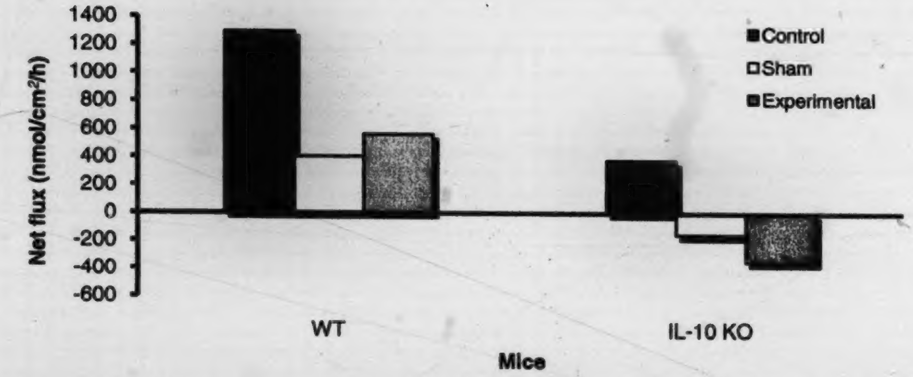
The mucosal-to-serosal glucose flux was statistically significantly lower in the IL-10 KO mice compared to the WT mice ($p < 0.001$). There was no statistically significant difference between control, sham and ileocolonic anastomosis groups in both, WT and IL-10 KO mice.

Figure 4.3. **Serosal-to-mucosal ($J_{serosal}$) glucose flux in WT and IL-10 KO mice at 6 weeks after surgery.**



There was a statistically significant difference between the WT and IL-10 KO mice in the serosal-to-mucosal glucose flux (p = 0.050). There was no statistically significant difference between control, sham and ileocolonic anastomosis groups in both, WT and IL-10 KO mice.

Figure 4.4. **Net mucosal-to-serosal (J_{net}) glucose transport in WT and IL-10 KO mice at 6 weeks after surgery**



The net glucose transport was significantly lower in the IL-10 KO mice then the WT mice (p = 0.05). There was a trend towards reduced glucose transport in animals that underwent both sham operation and ileocolonic anastomosis, which was present in WT and IL-10 KO mice. This trend was not statistically significant.

4.6. REFERENCES

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Chapter 5. Mucosal and Systemic Cytokine Response Following Ileocolonic Anastomosis

5.1. INTRODUCTION

Immunology of Crohn's Disease

Although the immune system plays a crucial role in the pathogenesis of Crohn's disease (CD), CD is not considered to be an autoimmune disease because the immune response is not directed against self-antigens. In CD, the inflammation of the bowel results from an exaggerated and inappropriate immune response directed against the resident gastrointestinal bacteria.

The immune system is composed of two arms of defense, innate and adaptive immunity. Innate immunity provides the first line of defense based on broad recognition of potentially dangerous antigens. The hallmarks of this broad recognition are the pattern recognition receptors (PRR), the trans-membrane Toll-like receptors (TLRs) and the intra-cytoplasmic nucleotide-binding-oligomerization-domain proteins (NODs), which identify groups of pathogens based on conserved, pathogenic structures termed pathogen-associated molecular patterns (PAMPs).[1] The adaptive immunity recognizes specific pathogens rather than broad groups and is capable of forming memory cells for more efficient pathogen clearance upon subsequent encounters. In theory, the two systems are distinct entities with different properties. In reality, the two systems are very tightly interwoven and are dependent on each other for the most efficient protection against pathogen invasion. The engagement of innate mechanisms such as binding of pathogenic components to TLRs initiates a cascade of events that leads to activation of adaptive immunity. Cross talk between the two arms of the immune system relies on a complex orchestration of immune cells, intra-cellular signaling, chemokines and cytokines. Essentially, the innate immunity allows time for the adaptive immunity to prepare for the ultimate elimination of pathogenic invaders.

Disturbance in adaptive immunity was initially recognized as being pathogenic in CD. This disturbance is T cell mediated bowel injury due to increased differentiation of the naïve T cells into type 1 T helper cells (Th1), producing pro-inflammatory cytokines interleukin-12 (IL-12) and interferon-gamma (IFN-γ).[2, 3] Tumor necrosis factor (TNF) produced by recruited macrophages is also recognized as a prototypical CD cytokine and antibodies against this cytokine have proven to be an efficacious therapy in some subgroups of patients with CD.[4-6] Advances in molecular technology and mass scanning of the human genome for potential gene polymorphisms in the context of inflammatory bowel disease (IBD) promise to further expand our knowledge of CD pathogenesis, and have led to new concepts in our understanding of the disease.

T helper 17 cell (Th17), a recently discovered component of the adaptive immunity, which expresses interleukin-17 (IL-17), is also now recognized to play a role in CD pathogenesis.[7] Cytokines interleukin-6 (IL-6) and transforming growth factor beta (TGF-β) induce the differentiation of naïve T cells into Th17 cells.[8, 9] Interleukin-23 (IL-23) is required for their maintenance.[9] CD patients have been shown to have increased numbers of IL-17 producing cells in the bowel wall, increased mucosal expression of IL-17 and elevated IL-17 concentrations in the serum.[7] Elevation of IL-17 is highest in patients with active disease and is absent in controls (ischemic and infectious colitis).[7] While the exact mode of action of IL-17 awaits elucidation, evidence to date supports IL-

17 as a mediator of pro-inflammatory response. This evidence includes productionof pro-inflammatory cytokines IL-6, interleukin-8 (IL-8), interleukin-1-beta (IL-1β) and TNF in the presence of IL-17.[10-12]

Recently, the focus of CD pathology has also extended from adaptive to innate immunity. Abnormal PRR expression and innate immune cells have now been implicated to play a central role in CD pathogenesis.[13-15] For instance, in active CD TLR3 has been shown to be down-regulated on the intestinal epithelial cells, while TLR4 shows increased expression.[13] *CARD15* polymorphism is one of the most studied genetic aberrancies associated with CD. The gene product of *CARD15* is a cytosolic PRR NOD2. NOD2 binds muramyl dipeptide, a component of bacterial peptidoglycan, and activates the transcription nuclear factor kappa B (NF-κB), resulting in among others in secretion of α-defensin by the intestinal Paneth cells and induction of pro-inflammatory cytokines by antigen presenting cells.[14-16] NOD2 mutations are present in approximately 20% of patients with CD and correlate with more aggressive disease with increased requirements for surgery and subsequent re-operations.[17, 18]

Dendritic cells bridge the innate and adaptive immunity as they initiate adaptive responses through antigen presentation to naïve T cells. In IBD dendritic cells have been implicated in incorrect recognition of commensal bacteria as pathogenic and activation of effector T cells rather than regulatory T cells.[19] In addition, non-professional antigen presenting cells (namely intestinal epithelial cells) can successfully induce naïve CD4+ T cells to differentiate into Th1 and Th17 cells during inflammation. In the homeostatic state these cells lack the necessary co-stimulatory signal and induce anergy. [20-22]

In summary, the bowel inflammation in CD results from alterations in the immune system operating on multiple levels in response to commensal gastrointestinal flora. An inappropriate response to resident bacteria at the innate immunity level and subsequent activation of the adaptive immunity leads to tissue injury, which perpetuates the disease process.

Immunology of Wound Healing

Surgery causes acute tissue injury and stimulates the healing process. The immune system plays a pivotal role in normal tissue repair. It is reasonable to speculate that in the context of CD, activation of the immune system in wound healing following surgery may have a compounding effect on the already dysregulated immune system. Wound healing is an extremely complex process involving wide spectrum of cellular components and inflammatory mediators. Wound healing is classically divided into three phases: (1) inflammatory phase (2) proliferative phase and (3) remodeling phase.[23] Although these phases are routinely described as separate entities in the literature, in reality there is no clear distinction between them. Immediately following injury hemostasis begins, where vasoconstriction, platelet aggregation and activation of the clotting cascade stops the bleeding.[24] The fibrin clot that is generated also serves as a scaffold for further repairs. Once hemostasis is established, local vessel dilation and release of chemoattractants facilitate migration of neutrophils and macrophages to the site of injury.[24] Neutrophils and macrophages synthesize mediators that stimulate further repairs, while clearing debris such as dead cells, foreign bodies and bacteria through phagocytosis.[24] In addition, macrophages produce the growth factors and cytokines that lay the foundation for the next phase.

The proliferative phase is characterized by angiogenesis, fibroblast proliferation and deposition of the extra-cellular matrix (collagen and proteoglycans). It is also during this phase that the wound becomes epithelized. In skin wounds the epithelium is regenerated from wound edges and skin appendages.[25] The re-epithelization of the gastrointestinal tract is unique and consists of two processes: restitution and regeneration.[26] Epithelial restitution, involving migration of epithelial cells from the wound edges, occurs within minutes to hours of injury. It restores epithelial continuity and maintains epithelial barrier against luminal contents.[26] Epithelization is completed through epithelial cell proliferation, differentiation and maturation.[27] The transition to the final stage of wound healing takes place when equilibrium between extracellular matrix synthesis and degradation has been achieved. This signifies the remodeling phase has been established. During this stage the extracellular matrix undergoes re-organization leading to increased tensile strength.[24]

TGF-β, TNF, IFN-γ, IL-1 and IL-6 appear during wound healing and overlap with the cytokines implicated in the pathogenesis of CD.[23] TGF-β in particular is a pivotal cytokine in wound healing. It appears very early, being released by platelets, macrophages, fibroblasts and endothelial cells.[25] It serves a multitude of functions including chemotaxis, stimulation of angiogenesis, fibroblast proliferation, synthesis of collagen and fibronectin and increased expression of metalloproteinase inhibitors.[23, 25] In the bowel, TGF-β is an essential mediator of mucosal repair and of epithelial cell restitution.[27, 28] IL-1 and TNF are also released early in wound repair (mainly by macrophages) and play a role in chemotaxis, endothelial cell migration during angiogenesis and fibroblast proliferation.[25] IL-6 is released by macrophages, and fibroblasts and it functions as a chemoattractant for further migration of macrophages and fibroblasts to site of injury and it up-regulates metalloproteinase inhibitors.[23] IFN-γ is produced by macrophages and it is involved in activation of monocytes.[23]

The immunological responses involved in the pathogenesis of CD and the process of wound healing are very complex and patients with CD who require surgical intervention experience both processes. In addition, ileocolonic anastomosis results in altered bacterial antigen load in the neo-terminal ileum (presented in Chapter 3).

It is possible that all these processes are interconnected and augment each other, contributing to the observed rapid post-operative recurrence of inflammation. The aim of our study was to clarify this connection by examining the mucosal and systemic immune changes that follow ileocolonic anastomosis in IL-10 KO mice susceptible to develop CD-like bowel inflammation.

Our objectives were to examine the expression of pro-inflammatory and anti-inflammatory cytokines following ileocolonic anastomosis in the mucosa of the neo-terminal ileum and spleen. Mucosal messenger RNA (mRNA) levels of IL-6, IL-12, IL-17, IFN-γ, TNF and TGF-β were measured in the neo-terminal ileum using relative quantification real time polymerase chain reaction (qRT-PCR) at 6 and 15 weeks after surgery. To assess the systemic immune response we isolated splenocytes at 6 and 15 weeks after ileocolonic anastomosis and measured IFN-γ and IL-17 release after stimulation with endogenous bacterial antigens.

5.2. MATERIALS AND METHODS

Animals and Surgeries

The animals and surgeries used were previously described in the Materials and Methods section of Chapter 2.

RNA Isolation

At 6 weeks and 15 weeks post-operatively animals were euthanized by cervical dislocation. A 2 cm segment of the terminal ileum was collected immediately proximal to the ileocecal junction in the control and sham operated animals, and proximal to the ileocolonic anastomosis in the experimental animals. Each segment was washed with 3 mL of phosphate buffered saline pH 7.2 [INVITROGEN, Carlsbad, CA], homogenized with a sterile razor blade, snap frozen in liquid nitrogen in 500μL of Trizol® Reagent [INVITROGEN, Carlsbad, CA] and stored at -70°C. The RNA was isolated using a commercial kit, RNeasy® Plus Mini kit [QIAGEN, Germantown, MD]. For the details of RNA isolation please refer to Chapter 3, Materials and Methods section.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The cDNA was generated using the High Capacity Reverse Transcription Kit with RNase inhibitor [Applied Biosystems, Foster City, CA] as per manufacturer's instructions. For details please refer to Chapter 3, Materials and Methods section.

Relative Quantification Real Time Polymerase Chain Reaction

The relative quantification real time PCR was analyzed using a 7900 HT Fast Real-Time PCR System [Applied Biosystems, Foster City, CA]. The TaqMan primers and probes for IL-6 (Mm 01210733_m1), IL-10 (Mm 01288386_m1), IL-12a (Mm 00434169_m1), IL-17a (Mm 00439619_m1), IFN-γ (Mm 00801778_m1), TNF (Mm 00553260_g1), TGF-β (Mm 00441729_g1) and β-actin (Part number: 4352341E) were purchased from Applied Biosystems [Foster City, CA] and the Gene Expression Assay was set up following manufacturer's instructions. In brief, each reaction well contained 1 μL TaqMan Gene Expression primer, 1 μL of β-actin internal control, 2 μL of cDNA to be tested (200 ng of original RNA), 6 μL RNase free water, and 10 μL of TaqMan Universal PCR Master Mix (2x) without AmpErase UNG. The PCR thermal cycling conditions comprised of: 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C and 40 cycle of (15 sec at 95°C + 1min at 60°C). Each reaction well contained β-actin as the internal control and each sample was analyzed in triplicate. The wild type control animals (no surgery) were used as a reference group so that the mRNA levels of all other groups of mice were expressed as a relative ratio against WT control mice. Relative quantity (RQ) was used to express the results and the data was analyzed using SDS RQ Manager software [Applied Biosystems, Foster City, CA], based on threshold cycle (C_t) values for the internal control and cytokine of interest. RQ was calculated using the following equations:

$$RQ = 2^{-\Delta\Delta Ct}$$

where *RQ* is relative quantification, $\Delta\Delta C_t = \Delta C_t$ test group - ΔC_t reference group (WT control group) and $\Delta C_t = C_t$ target – C_t internal control (β-actin). C_t was defined as the PCR cycle at which the increase in reporter fluorescence signal was first detected to be above baseline.

Splenocyte Stimulation

Mice were euthanized at 6 and 15 weeks post-operatively by cervical dislocation and spleens were collected. A single-cell suspension of splenic cells in complete RPMI [GIBCO, Carlsbad, CA] with 10% fecal bovine serum was prepared as previously described by Sydora *et al.*[29] In brief, spleens were minced between frosted-end sterile microscopic slides and depleted of red blood cells by osmotic lysis. 2 x10^5 cells/well suspensions were placed in 96-well plates in quadruplicate and incubated at 37°C in humidified incubator at 5% CO_2 in the presence of: (1) RPMI medium (negative control), (2) plate-bound anti-CD3ε antibody clone 145-2C11 (positive control) [PharMingen Canada, Mississauga, ON], (3) fecal sonicates from germ-free mice lacking bacterial antigens (50 μg/mL protein concentration) and (2) fecal sonicates from specific pathogen free mice containing bacterial antigens (50 μg/mL protein concentration). The fecal sonicates were prepared under sterile conditions as previously described by Sydora *et al.*[30] Following 48-hour incubation, plates were centrifuged for 5 min at 100*g* and IFN-γ and IL-17 levels in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA).

Enzyme Linked Immunosorbent Assay

Interferon-γ

Splenic lymphocyte production of IFN-γ was determined using Mouse IFN-γ DuoSet® ELISA Development System [RnDSystems, Minneapolis, MN] following manufacturer's directions. In brief, 96-well high-protein-binding ELISA plates (Costar, Cornins Inc., Coming, NY) were coated overnight with 100 μl/well of rat anti-mouse IFN-γ capture antibody provided in the kit, which was diluted in PBS [137mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2] to a final working concentration of 4.0 μg/mL. Next, the plate was blocked with 300 μl/well of 1% bovine serum albumin (BSA) in PBS blocking buffer with 0.05% NaN_3 for 1 hour. One hundred μl of sample or standard was added to each well and incubated for 2 hours at room temperature. Each sample/standard was applied in duplicate. Prior to application, samples collected from stimulation with anti-CD3ε antibody and fecal lysates with bacterial antigens were further diluted in RPMI medium to 1:100 and 1:10, respectively, to accommodate the standard curve. Standards were supplied with the kit and prepared according to manufacturer's specifications. Serial dilutions of the stock standard were performed in Reagent Diluent [0.1% BSA, 0.05% Tween 20, 20mM Trizma base, 150 mM NaCl, pH 7.2] such that the standard curve contained the following concentrations: 0, 31.25, 62.5, 125, 250, 500, 1000, and 2000, pg/mL. One hundred μl of biotinylated goat anti-mouse IFN-γ detection antibody diluted to working concentration of 400 ng/mL in Reagent Diluent was added to each well and incubated for 2 hours at room temperature. In between application of each solution the plates were washed three times with wash buffer (0.05% Tween 20 in PBS, pH 7.2). The plates were developed with 100 μl/well of Streptavidin-horse radish peroxidase (1:200 dilution) [RnD Systems, Minneapolis, MN] for 20 min, followed by incubation in direct light for 20 min with 100 μl/well of Substrate Solution [1:1 mixture of Color Reagent A (tetramethylbenzidine) and Color Reagent B (hydrogen peroxide), [RnDSystems, Minneapolis, MN] and stopped with 50 μl of Stop Solution (2N H_2SO_4). The plates were read at 450 nm in the Wallac Victor 2 1420 plate reader [Perkin Elmer, Wellesley, MA] and the cytokine concentration in each sample was determined using the standard curve.

Interleukin-17

Mouse IL-17 ELISA kit was used [eBioscience, San Diego, CA] following manufacturer's instructions. Ninety six-well high-protein-binding ELISA plates (Costar, Cornins Inc., Coming, NY) were coated overnight with 100 μl/well of purified anti-mouse IL-17 antibody diluted 1:250 in Coating Buffer provided in the kit (final working concentration 4 ng/mL). Ninety μl of sample or standard was added to each well and incubated for 2 hours at room temperature. Each sample/standard was applied in duplicates. Samples collected from stimulation with anti-CD3ε antibody were diluted 1:10 in RPMI medium prior to application to accommodate the standard curve. Standards were supplied with the kit and prepared according to manufacturer's specifications. Serial dilutions of the stock standard were performed in Assay Diluent such that the standard curve contained the following concentrations: 0, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 pg/mL. 100 μl of 4 ng/mL of biotin-conjugated anti-mouse IL-17A in Assay Diluent was added to each well and incubated for 1 hour at room temperature. In between application of each solution the plates were washed five times with wash buffer (0.05% Tween 20 in PBS, pH 7.2). The plates were developed with 100 μl/well of Avidin-HRP (1:250 dilution) [eBioscience, San Diego, CA] incubated for 30 min and washed seven times with washing buffer, followed by 15 min incubation in the dark with 100 μl/well of tetramethylbenzidine substrate solution and stopped with 50 μl of 2 N H_2SO_4. The plates were read at 450nm in the Wallac Victor 2 1420 plate reader [Perkin Elmer, Wellesley, MA] and the cytokine concentration in each sample was determined using the standard curve.

Statistical Analysis

Statistical analysis of ELISA data was performed using Stata 10.0 [Statacorp LP, College Station, TX]. Concentrations of IFN-γ and IL-17 were expressed as means ± SEM. At each time point all groups were compared by the Kruskal-Wallis test. If the null hypothesis of the Kruskal-Wallis test was rejected, a multiple pair-wise comparison was performed using Mann-Whitney U. Bonferroni's correction was applied where we considered a p-value to be statistically significant if it was less than 0.05 divided by the number of groups being compared. Statistical analysis of relative quantification was carried out using SDS RQ Manager software [Applied Biosystems, Foster City, CA] designed for qRT-PCR data analysis. For all time points the WT control group (no surgery) served as a reference group (RQ = 1). Data were expressed as RQ ± SD. All figures were generated using Excel Microsoft 2004 [Redmond, WA].

5.3. RESULTS

Mucosal Cytokine Expression

TNF

Mucosal TNF mRNA was elevated at 6 weeks after surgery in the IL-10 KO mice that underwent ileocolonic anastomosis with RQ value of 6.08 ± 0.34 (Figure 5.1). No increase in TNF was detected for all other groups of WT and IL-10 KO mice at 6 and 15 weeks after surgery and IL-10 KO mice with ileocolonic anastomosis at 15 weeks.

TGF-β

TGF-β mRNA expression was increased in IL-10 KO mice that underwent sham operation (RQ = 2.19 ± 0.20) and ileocolonic anastomosis (RQ = 4.48 ± 0.19) at the 15-week time point (Figure 5.2). There was no increase in TGF-β mRNA in all WT and IL-10 KO groups at 6 weeks and in all WT groups and the IL-10 KO control group at the 15-week time interval.

Other Cytokines

We were unable to detect mRNA for IFN-γ, IL-6, IL-12, and IL-17 at the 6- and 15-week time points. In the WT mice, there were no detectable levels of IL-10 at the same time points.

Systemic Cytokine Expression

Interferon-γ

Wild Type Mice

At 6 weeks after surgery, in all three groups of WT mice (control, sham and ileocolonic anastomosis), splenocyte stimulation with endogenous bacterial antigens did not lead to release of IFN-γ (Figure 5.3A). There was, however a strong response to stimulation with anti-CD3ε antibody (positive control) in all WT animals, indicating the viability of splenocytes (Figure 5.3B).

At the 15-week time interval, similar to the 6-week time interval, the WT control group continued to demonstrate no systemic IFN-γ expression in splenocytes. In contrast, WT animals that underwent sham or ileocolonic anastomosis operation had increase in IFN-γ expression by splenocytes stimulated with fecal sonicates containing bacterial antigens (Figure 5.3A). In the sham-operated group the increase in IFN-γ expression was 74.11 ± 28.55 pg/mL, but did not reach statistical significance when compared to WT control group. In the ileocolonic anastomosis group, however, the increase in IFN-γ expression was 237.13 ± 75.35 pg/mL and was statistically significant relative to the WT control group ($p = 0.024$). In both groups (sham operation and ileocolonic anastomosis operation) at 15 weeks, stimulation with fecal sonicates devoid of bacterial antigens did not result in IFN-γ expression while stimulation with anti-CD3ε antibody remained strongly positive implying that the systemic response was related to luminal bacterial antigens (Figure 5.3B).

IL-10 KO Mice

In the IL-10 KO mice at the 6-week time interval, IFN-γ was increased in all three groups, (control 1134.17 ± 503.18 pg/mL, sham 1261.25 ± 947.36 pg/mL, ileocolonic anastomosis 896.78 ± 673.10 pg/mL) in response to stimulation with fecal bacterial antigens but there was no statistically significant difference between the groups ($p = 0.790$) (Figure 5.4). Stimulation with fecal sonicates devoid of bacterial antigens did not result in IFN-γ increase in all three groups, again implying the response was related to the fecal bacterial antigens.

At 15 weeks, there was no change from the 6-week time interval in IFN-γ expression in the IL-10 KO control (960.80 ± 668.79 pg/mL) and IL-10 KO sham

(1112.25 ± 694.02 pg/mL) groups (Figure 5.4). In contrast, stimulation with bacterial antigens resulted in a significant IFN-γ increase (6027.50 ± 1777.89 pg/mL) in the ileocolonic anastomosis group. This increase was statistically significant relative to the IL-10 KO ileocolonic anastomosis group at the 6-week time point ($p = 0.004$), and the IL-10 KO control and sham groups at 15 weeks ($p = 0.029$ and $p = 0.037$ respectively).

Interleukin-17

Wild Type Mice

Stimulation of splenocytes from WT mice with bacterial antigens did not result in IL-17 release in all surgical groups (control, sham and ileocolonic anastomosis) at both the 6 and 15-week time intervals. Stimulation of the same groups with anti-CD3ε antibody resulted in modest increase in IL-17 expression, which was lower than the stimulation with anti-CD3ε antibody of splenocytes from IL-10 KO mice at 6 and 15 weeks. In all WT groups no IL-17 was released in response to stimulation with germ-free fecal sonicates.

IL-10 KO Mice

In the IL-10 KO mice, splenocyte stimulation with bacterial antigens at 6 weeks after surgery resulted in IL-17 release in the three surgical groups (control 191.09 ± 48.64 pg/mL, sham 229.19 ± 100.02 pg/mL, experimental 265.60 ± 176.34 pg/mL) but there was no statistical difference between groups ($p = 0.458$) (Figure 5.5). At 15 weeks, IL-17 release in response to bacterial antigens in IL-10 KO control and sham group was unchanged from the 6-week time point (control 217.23 ± 99.48 pg/mL and sham 301.22 ± 110.24 pg/mL) (Figure 5.5) and there was no statistical difference between the two groups ($p = 0.465$). In the IL-10 KO mice that underwent ileocolonic anastomosis, stimulation of splenocytes with bacterial antigens resulted in a significant increase in the IL-17 secretion of 603.57 ± 81.42 pg/mL (Figure 5.5). This increase was significant in relation to the IL-10 KO ileocolonic anastomosis group at the 6-week time point ($p = 0.032$) and the IL-10 KO control group at 15 weeks ($p = 0.017$) but not compared to the IL-10 KO sham group at 15 weeks ($p = 0.078$).

5.4. DISCUSSION

We have shown that ileocolonic anastomosis in IL-10 KO mice resulted in increased mucosal mRNA levels of TNF and TGF-β at 6 and 15 weeks after surgery, respectively. Both these cytokines play an important role in bowel inflammation associated with CD and surgical wound healing. For instance, TNF is involved in chemotaxis and angiogenesis during wound healing while in CD it is a potent mediator of bowel inflammation.[4, 25] There are couple plausible explanations for the observed elevation in TNF in our study. TNF may be increased secondary to post-surgical wound healing at the anastomosis. This explanation is less likely given that TNF was not elevated in mice undergoing sham operation (small bowel transection and re-anastomosis). TNF increase was only observed in the immunologically deficient mice and only after ileocolonic anastomosis suggesting that the combination of an increased bacterial colonization of small bowel (demonstrated in Chapter 3) and a susceptible individual (IL-10 KO mouse) may be responsible for this elevation. This finding however, cannot be readily related to human disease as the IL-10 KO mice lack IL-10, an anti-inflammatory cytokine produced mainly by Tr1 regulatory cells and known to suppress TNF.[31, 32]

Mucosal TGF-β mRNA level was elevated two-fold and four-fold in the IL-10 KO mice following the sham and ileocolonic anastomosis operations respectively. The presence of TGF-β can be both protective and pathogenic. In its protective role, TGF-β is an essential cytokine for wound healing and it is a ubiquitous mediator of the proliferative phase.[23] Additionally, TGF-β plays a critical role in the mucosal homeostasis being produced by CD4+CD25+ regulatory T cells and in inducing the peripheral differentiation of naïve T cells into regulatory T cells.[33] Regulatory T cells are present throughout the gastrointestinal lamina propria and participate in oral tolerance and they can suppress colitis through inhibition of the Th1 response. [33-35] There is also evidence for TGF-β's pathogenic role specifically in the setting of CD. First, TGF-β has been shown to be a crucial mediator of naïve T cells differentiation into a recently recognized pro-inflammatory Th17 lineage, which has been implicated in pathogenesis of CD.[7, 8] Second, increased fibrogenesis causing strictures in CD patients has been attributed to over-expression of TGF-β.[36] In our study we demonstrated at the 15-week time point increased mucosal TGF-β mRNA and systemic IL-17 release in response to bacterial antigens both present in the IL-10 KO mice after ileocolonic anastomosis. It is possible the two findings are linked. The almost universal disease recurrence at the anastomosis after ileocecal resection may be the result of TGF-β production at the surgical anastomosis in the context of wound healing driving a Th17 mediated inflammatory response. This response may be further perpetuated by the increased bacterial colonization around the anastomosis after the loss of ileocecal valve. To further address the role of TGF-β in post-operative fibrosis we examined the neo-terminal ileum for collagen deposition after ileocolonic anastomosis and the results for this experiment are discussed in Chapter 6. Overall, TGF-β seems to play a dual role, being both an anti and pro-inflammatory mediator. While the exact mechanism for the switch between the two is being elucidated, our study suggests that this mechanism may be an important factor in the post-operative immune changes that lead to CD recurrence.

Mucosal mRNA for IFN-γ, IL-6, IL-12, and IL-17 was undetectable at 6 and 15 weeks for all groups and TNF was also undetectable at 15 weeks. This does not necessarily imply that the cytokine encoded by a given mRNA is completely absent. The lack of detection is not likely due to the technical aspect of the qRT-PCR protocol as there was good expression of the internal control β-actin, which was included in every reaction. The lack of mRNA detection perhaps illustrates the limitation of measuring mRNA instead of the final protein. Messenger RNA represents a very transient and dynamic intermediate state of protein synthesis. There are a number of post-transcriptional mechanisms within the cell that moderate the rate of mRNA stability by either promoting translation or rapid decay of the message. The adenine and uridine-rich element at the 3' untranslated region is an example of such mechanism specific to cytokines, which can promote rapid decay of mRNA.[37] At any given time mRNA can be translated at polysomes, stored in stress granules for rapid release when needed or alternatively, be degraded in processing bodies.[37] This uncertain state of mRNA at any given time point makes it a surrogate of unknown reliability for the true presence of cytokines. Measuring cytokine protein secretion directly using ELISA is likely to produce a much better estimate.

Systemic immune studies of splenocyte stimulation with endogenous bacterial flora demonstrated increased release of pro-inflammatory cytokines (IFN-γ and IL-17) in IL-10 KO mice that underwent ileocolonic anastomosis. At 6 weeks after surgery all three groups (control, sham and ileocolonic anastomosis) of IL-10 KO mice showed elevated IFN-γ and IL-17. This is expected due to the underlying active colonic inflammation that was present in our IL-10 KO animals during the course of the study and the fact that active intestinal inflammation leads to loss of tolerance to endogenous microbes.[38] At 15 weeks after surgery, IL-10 KO mice that underwent the ileocolonic anastomosis had a further marked increase of IFN-γ and IL-17 above this background level, which was absent in the sham-operated animals, suggesting that this increase may be driven by bacterial flora changes described in Chapter 3.

At first glance, the relevance of this observation to CD patients might be questioned due to IL-10 deficiency in our animal model. IL-10 is known to suppress Th1 differentiation and hence IFN-γ.[39] However, a similar increase in IFN-γ was observed in the WT mice that do express IL-10 where we did not expect a systemic response. WT mice following the sham and experimental operations mounted a systemic response to endogenous bacterial antigens at 15 weeks after surgery. There are a number of non-mutually exclusive, explanations for the observed increase in IFN-γ in the operated animals. The surgical transection of bowel introduces a transient yet major breach in epithelial integrity allowing for massive bacterial translocation and subsequent immune response. This immune response can lead to formation of highly specific, long-lived pool of memory T-cells that even in the absence of co-stimulation can generate a more efficient and a higher magnitude response upon re-encounters with the same antigens.[40] In fact, bowel surgery may leave a long-lasting imprint in the immune system even in healthy individuals that can become pathological in CD patients. It is difficult to speculate if the memory T cell generation results from transient bacteremia or local antigen presentation at the bowel wall or both, and also what effect routine pre-operative antibiotic administration would have on these processes. Alternatively, the observed systemic response may be due to ongoing generation of effector T cells (Th1 and Th17 cells), resulting from ongoing antigen presentation of the increased bacterial antigens in the neo-terminal ileum by the extensive gut associated lymphoid tissue that is most prominent within the terminal ileum. Perhaps the sudden bacterial change triggers the activation of normally hypo-reactive dendritic cells or engages other cells such as the intestinal epithelial cells in antigen presentation leading to an immune response. There also may be other possible entry points for luminal bacterial antigens. For instance, a recent study using confocal endomicroscopy revealed cell-sized gaps in both human and murine epithelium within the terminal ileum.[41] These gaps are thought to represent a normal enterocyte shedding process and in healthy state, the epithelial barrier remains intact due to gap filling with a yet-unidentified impermeable substance.[42] The exact role of the gaps in inflammatory states remains unknown, however, in mice TNF has been shown to cause 27-fold increase in cell shedding and loss of barrier function. [41]

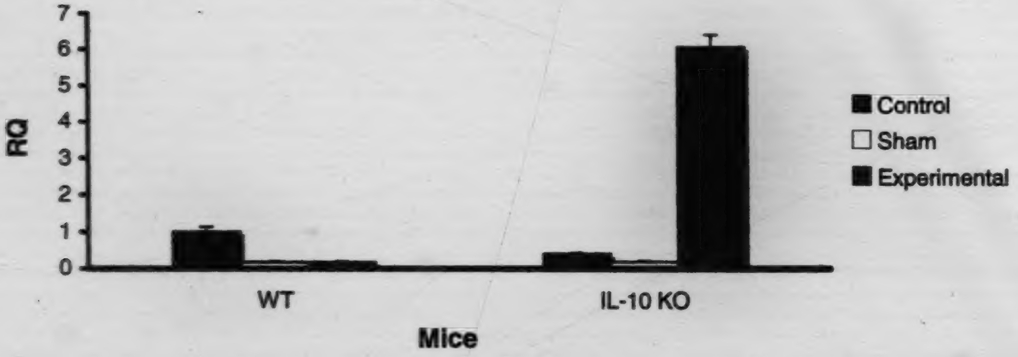
To clarify our observation of increased cytokine secretion a worthwhile future experiment would be to determine the exact composition of the isolated splenocytes using flow cytometry. This would allow for identification of the cell source of the cytokines, a first crucial step in elucidating the mechanism behind our findings of increased cytokine expression.

5.5. CONCLUSION

In this study we have investigated changes that occur after ileocolonic anastomosis. Bowel surgery involves a cascade of highly dynamic processes, which could go beyond the frame of this thesis if we wanted to investigate them it their entirety. In our study we have focused on cytokine changes after ileocolonic anastomosis and have demonstrated that in the IL-10 KO mice ileocolonic anastomosis leads to increased mucosal expression of mRNA for TNF at the 6th post-operative week and TGF-β at the 15th post-operative week. We also showed that at the 15-week time point, WT and IL-10 KO mice that underwent ileocolonic anastomosis had increased systemic release of IFN-

γ in response to stimulation with endogenous bacterial antigens while IL-17 expression was elevated in IL-10 KO mice only. In summary, ileocolonic anastomosis results in mucosal and systemic cytokine changes. Future studies using different murine models and measuring panels of secreted cytokines rather than mRNA and more importantly, studies of cytokine expression in patients with ileocolonic anastomosis will be valuable in further elucidating the immune processes present in the post-operative period. A synthesis of these processes together may offer an explanation for the observed rapid post-operative disease recurrence.

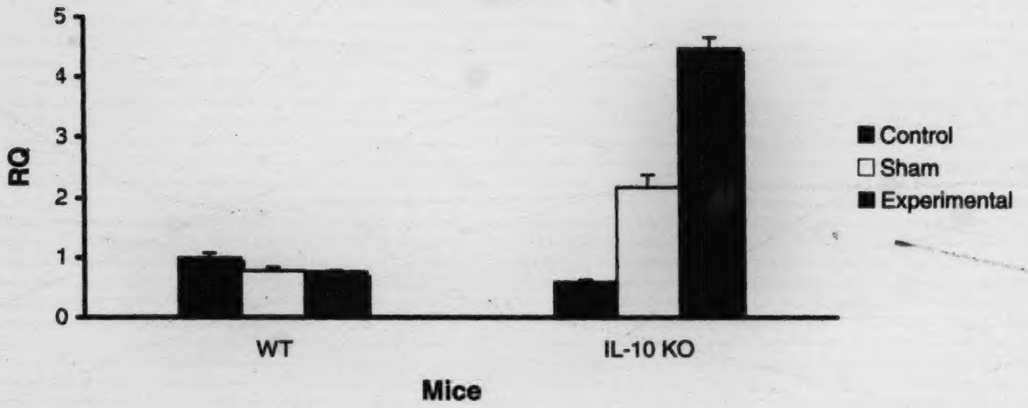
Figure 5.1. Mucosal TNF mRNA expression in IL-10 KO mice at 6 weeks after surgery



At 6 weeks after surgery, there was a six-fold increase in the mucosal TNF mRNA expression in IL-10 KO mice that underwent ileocolonic anastomosis operation.

RQ = relative quantity (in relation to WT control; RQ = 1); experimental = ileocolonic anastomosis.

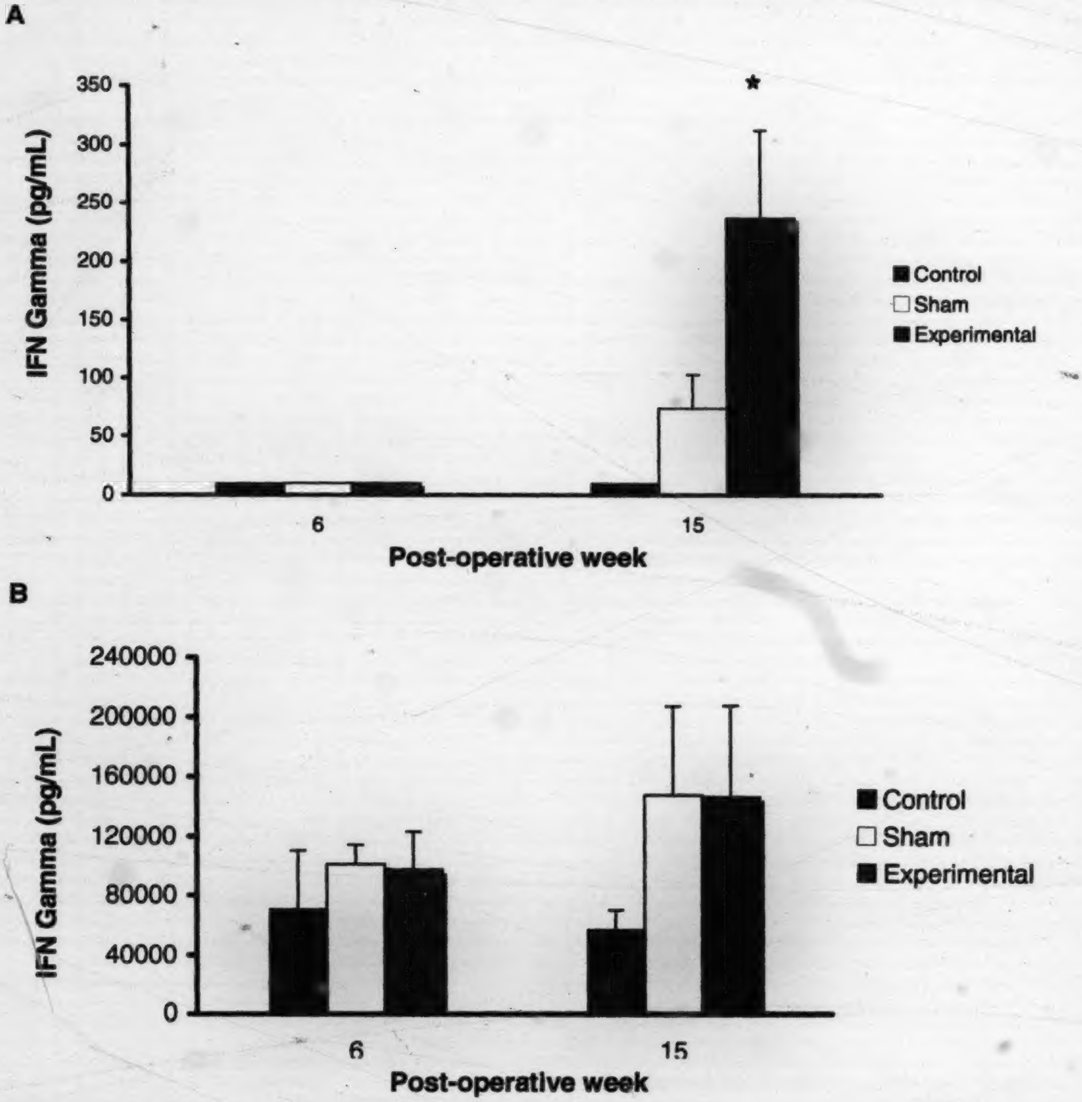
Figure 5.2. **Mucosal TGF-β mRNA expression in WT and IL-10 KO mice at 15 weeks after surgery**



At 15 weeks after surgery, there was a four-fold and two-fold increase in the mucosal TGF-β mRNA expression in IL-10 KO mice that underwent sham and ileocolonic anastomosis operations respectively.

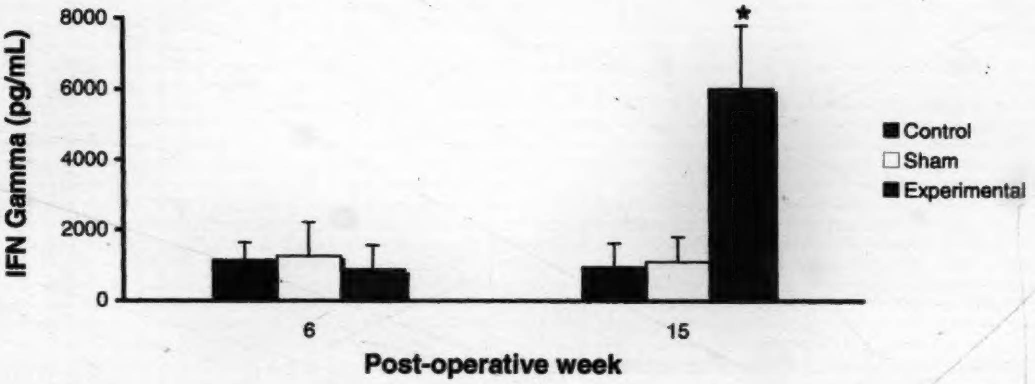
RQ = relative quantity (in relation to WT control; RQ = 1); experimental = ileocolonic anastomosis.

Figure 5.3. **IFN-γ release by splenocytes stimulated with endogenous bacterial antigens and anti-CD3ε antibody in WT mice at 6 and 15 weeks after surgery**



A. Upon stimulation with endogenous bacterial antigens (SPF-fecal lysates), IFN-γ release by splenocytes from WT mice was increased in animals that underwent the ileocolonic anastomosis, which was statistically significant (*p =0.024). **B.** There was a positive response at both time points to stimulation with anti-CD3ε antibody.

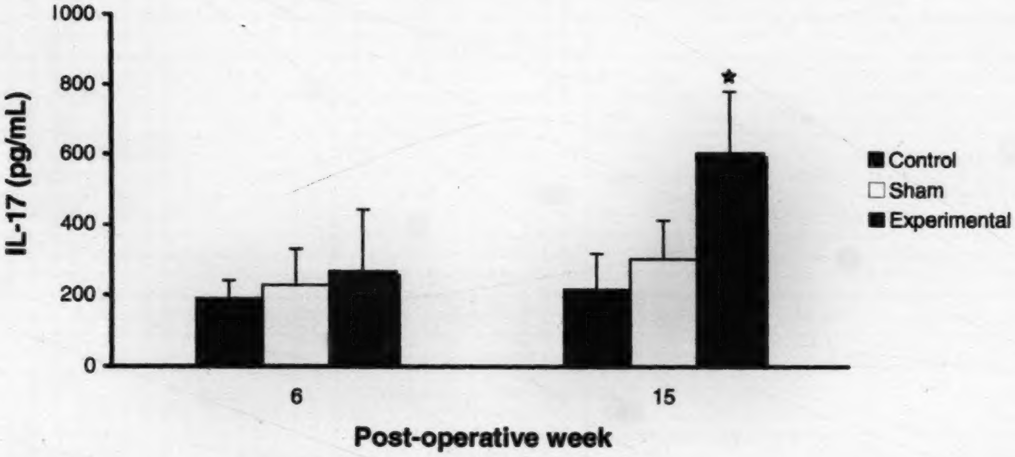
Figure 5.4. **IFN-γ release by splenocytes stimulated with endogenous bacterial antigens in IL-10 KO mice at 6 and 15 weeks after surgery**



Stimulation of splenocytes from IL-10 KO mice at 15 weeks after ileocolonic anastomosis with endogenous bacteria yielded a statistically significant increase in IFN-γ release when compares to IL-10 KO control and sham-operated animals (* $p < 0.05$).

Experimental = ileocolonic anastomosis.

Figure 5.5. IL-17 release by splenocytes stimulated with endogenous bacterial antigens in IL-10 KO mice at 6 and 15 weeks after surgery



Stimulation of splenocytes from IL-10 KO mice at 15 weeks after ileocolonic anastomosis with endogenous bacteria resulted in a statistically significant increase in IL-17 release when compared to IL-10 KO control animals (*p = 0.017).

Experimental = ileocolonic anastomosis.

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Chapter 6. Histological Inflammation and Collagen Content in the Neo-terminal Ileum

6.1. INTRODUCTION

Tissue Injury in Crohn's Disease

The diagnosis of CD is in part based on specific signs and symptoms and imaging studies (mainly endoscopy) but the definitive diagnosis comes from histological evaluation of biopsies obtained from the inflamed bowel.[1] The key diagnostic histological characteristics include inflammation involving the entire bowel wall thickness (transmural inflammation), discontinuous inflammatory lesions with normal intervening mucosa, mucosal erosions and ulcerations and lymphocytic and monocytic infiltration of the mucosa.[1] Presence of an epithelioid granuloma confirms the diagnosis, although granulomas have been inconsistently detected at rates from 15% to 60% depending on the type of specimen (resection specimen versus biopsy).[2, 3] These diagnostic criteria are very important in distinguishing CD from other etiologies of bowel inflammation and most importantly from ulcerative colitis as the optimal therapies can vary drastically between the diseases.

Tissue Injury in IL-10 KO Mice

The IL-10 KO mouse is a widely used model of CD where IL-10 gene deficiency leads to a number of pathological processes that highly resemble CD. Common characteristics shared by patients with CD and IL-10 KO mice include bowel inflammation and an exaggerated Th1 immune respose.[4, 5] Similar to the human disease, histological examination of the inflamed bowel from IL-10 KO mice shows multifocal transmural injury of the bowel wall leading to marked thickening, epithelial erosions and infiltration of the bowel wall with inflammatory cells, predominantly lymphocytes, macrophages and neutrophils.[5]

A number of scoring systems have been introduced to quantify the histological changes seen in the IL-10 KO mouse bowel.[5, 6] A validated histological inflammation score used in our lab is based on four parameters and is a modification of the previously described grading system by Saverymuttu *et al.*[6] This scoring system depicts maximal injury to be 10, which is a sum of four mucosal changes: (1) mucosal ulceration, (2) epithelial hyperplasia, and (3) lamina propria infiltration with neutrophils and (4) mononuclear cells (Table 6.1).[7] Using this scoring system, we assessed tissue injury at 6 and 15 weeks after ileocolonic anastomosis in WT and IL-10 KO mice. In addition, based on our finding of elevated mucosal TGF-β mRNA described in Chapter 5 we also performed histological examination of the neo-terminal ileum for post-operative collagen deposition using Masson's Trichrome collagen stain.

6.2. MATERIALS AND METHODS

Animals and Surgeries

Histopathological analysis was performed on tissues from the same mice that underwent ileocolonic anastomosis operation, sham operation and control treatment as previously described. For details please refer to Chapter 2, under the Materials and Methods section.

Histology

Tissue Injury Scoring

Mice were euthanized at 6 and 15-week time points by cervical dislocation. Two-centimeter segments of the ascending colon adjacent to cecum and 2 cm segments of terminal ileum proximal to the ileocecal junction (in control and sham animals) or the ileocolonic anastomosis were fixed in 10% neutral buffered formalin [SF-94-4 Formalde-Fresh solution, Fisher Scientific, Fair Lawn, NJ]. The segments were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H & E) for light microscopy examination. The slides were reviewed by a single pathologist familiar with the scoring system but blinded to the procedures the animals underwent. A validated histological grading of intestinal inflammation within the mucosa, described previously, was used to assign scores from zero (no injury) to 10 (maximal injury) based on mucosal ulcerations, epithelial hyperplasia and infiltration of lamina propria with neutrophils and mononuclear cells (Table 6.1) (Figure 6.1).[6, 7]

In addition, for the terminal ileum segments, this grading system was modified to include inflammatory cell (lymphocytic or neutrophilic) infiltration of the peri-ileal fat (Figure 6.2). The peri-ileal fat infiltration was scored on a two-point scale, zero being absence of infiltration and one point assigned in the presence of any infiltration. Consequently the maximum possible score for the ileal segments was 11 and the statistical analysis of ileal segments was performed separately.

Collagen Content

The ileum samples collected for the tissue injury scoring and embedded in paraffin were assessed for post-operative collagen deposition using Masson's Trichrome stain. The embedded tissues were sectioned at 5 μm. The samples were deparaffinized and hydrated with distilled water, incubated for 30 min at 60°C in filtered Bouin's solution (75 mL of 1.2% picric acid, 15 mL of 40% formaldehyde and 5 mL of glacial acetic acid) and then dried for 30 min at room temperature. The slides were washed under warm running tap water, stained with Masson's Trichrome stain (0.6 g of Chromotrope 2R, 0.3 g of Light green, 1.0 mL of glacial acetic acid, 0.8 g of phosphotungstic acid and 100 mL of distilled water) for 20 min, followed by 2 min incubation in 0.5% acetic acid and dehydrated in 100% ethanol. The Masson's Trichrome stain results in red muscle fibers and background and green collagen.

A three point collagen content scale was devised (Figure 6.3) and each segment of ileum was assigned a score from zero to two, zero being minimal collagen deposition and two being maximum thickness layer of collagen. A single pathologist who was blinded to the type of procedure the animals underwent assigned the score.

Statistical Analysis

Statistical analysis of all data was performed using Stata 10.0 [Statacorp, LP, College Station, TX]. All scores (tissue injury and collagen content) were expressed as means ± SEM. At each time point all groups were compared by the Kruskal-Wallis test. If the null hypothesis of the Kruskal-Wallis test was rejected, a multiple comparison was performed using Mann-Whitney U test to detect which pairs were statistically different. We considered a $p < 0.05$ to be statistically significant unless Bonferroni's correction for multiple pair-wise comparison was applied in which case p value less the 0.05 divided by the number of groups being compared was considered statistically significant. All figures were generated using Excel Microsoft 2004 [Redmond, WA].

6.3. RESULTS

Ileal Tissue Injury Score

Wild Type Mice

In the WT mice all of the ileal segments were normal at both the 6-week and 15-week time points independent of the type of surgery performed. The tissue injury scores were zero.

IL-10 KO Mice

There were changes in the tissue injury score noted in the IL-10 KO mice at 6 and 15 weeks in the ileocolonic anastomosis group only. At 6 weeks after ileocolonic anastomosis the mean tissue injury score 0.83 ± 0.71, which was not statistically significant relative to the IL-10 KO control (score = 0) and IL-10 KO sham-operated (score = 0) groups at the same time point (Figure 6.4A). The mean tissue injury score was higher in the IL-10 KO mice with ileocolonic anastomosis (1.17 ± 0.87) when the peri-ileal fat infiltration was included in the tissue injury score, however, it did not reach statistical significant relative to control ($p = 0.127$) and sham-operated ($p = 0.173$) IL-10 KO mice. The peri-ileal fat infiltration was present in 33% of animals at the 6-week time interval (Figure 6.4B). By 15 weeks the tissue injury score was reduced in the IL-10 KO mice with the ileocolonic anastomosis (0.38 ± 0.21) (Figure 6.4B).

Colonic Tissue Injury Score

Wild Type Mice

The colonic segments from WT mice were all normal at all time points regardless of the operation performed (scores of zero).

IL-10 KO Mice

In the IL-10 KO mice there was colonic inflammation present at both the 6-week and 15-week time points and in all three groups (control, sham and ileocolonic anastomosis). At 6 weeks the mean tissue injury scores for the control, sham and ileocolonic anastomosis groups were 5.57 ± 1.73, 4.33 ± 1.82 and 3.50 ± 1.49, respectively (Figure 6.5). There was no statistical difference between the groups at this time point ($p = 0.667$). At 15 weeks, the tissue inflammation was more severe in the IL-10 KO control group where all animals had the maximum score of 10 points (Figure 6.5). The animals that underwent the sham and ileocolonic anastomosis operations also had more severe colonic inflammation, however, the increase in the tissue injury score was more moderate. The mean tissue injury scores were 7.57 ± 1.45 for sham-operated group and 6.75 ± 1.34 for the ileocolonic anastomosis group (Figure 6.5). There was a statistically significant difference between the IL-10 KO sham-operated group ($p < 0.001$) and IL-10 KO ileocolonic anastomosis group ($p < 0.001$) compared to IL-10 KO control group but no significant difference was detected between the sham and ileocolonic anastomosis operations ($p = 0.120$).

Collagen Deposition

Wild Type Mice

There was increased collagen content within the bowel wall at 6 and 15 weeks after surgery noted in the WT mice that underwent sham and ileocolonic anastomosis operations. The mean collagen scores were 0.29 ± 0.20 for the WT control group, 1.00 ± 0.28 for the WT sham group and 1.67 ± 0.23 for the WT ileocolonic anastomosis group (Figure 6.6). There was no statistically significant difference between the WT control and WT sham-operated groups ($p = 0.141$). The WT ileocolonic anastomosis group relative to WT control group reached a statistical significance ($p = 0.004$), however, there no difference between the WT ileocolonic anastomosis and WT sham-operated animals (p = 0.224).

At 15 weeks post-operatively a decrease was noted in the collagen content among the WT mice in the sham group (0.68 ± 0.23) and ileocolonic anastomosis group (1.14 ± 0.28) (Figure 6.6). This decrease led to loss of the statistical difference detected between the groups at 6 weeks (p = 0.088).

IL-10 KO Mice

Similarly to the WT mice, sham and ileocolonic anastomosis operations in the IL-10 KO mice led to increased collagen content within the bowel wall. The increased collagen scores of 1.17 ± 0.34 for the sham-operated group and 1.40 ± 0.27 for the ileocolonic anastomosis group reached statistical significance relative to the IL-10 control group (p = 0.004 and p = 0.001 respectively) (Figure 6.7). There was no difference detected between the sham and ileocolonic anastomosis groups (p = 0.609). Matching for the procedure performed, there was no statistically significant difference in collagen score between the WT and IL-10 KO mice at 6 weeks after surgery (p = 0.141 for control, p = 0.652 for sham and p = 0.399 for ileocolonic anastomosis) (Figure 6.8A).

At the 15-week time interval, IL-10 KO sham-operated animals had a small reduction in the collagen content (1.00 ± 0.24), which remained statistically significant compared to the IL-10 KO control group (p = 0.008). In contrast, the IL-10 KO mice that underwent ileocolonic anastomosis showed an increase in the collagen score from 1.40 ± 0.27 (at 6 weeks) to 1.89 ± 0.15 (Figure 6.7). This increase was statistically significant when compared to both, the IL-10 KO control group (p = 0.002) and IL-10 KO sham-operated group (p = 0.010). In procedure-matched comparison between the WT and IL-10 KO mice at 15 weeks, the score for the IL-10 KO ileocolonic anastomosis group was significantly higher (p = 0.035) (Figure 6.8B).

6.4. DISCUSSION

In patients with CD, ileocolonic anastomosis leads to rapid disease recurrence with pathological mucosal changes in the neo-terminal ileum evident on endoscopy in 72% of patients within the first post-operative year.[8] The ileocolonic anastomosis in our study did not lead to rapid and diffuse inflammation of the neo-terminal ileum, as might be predicted based on the findings in patients with CD. Nevertheless there were some changes present on histology in the neo-terminal ileum of the IL-10 KO mice following ileocolonic anastomosis that were absent in the control and sham IL-10 KO groups and in all WT mice. These findings in conjunction with the increased colonization of the neo-terminal ileum with colonic bacteria (demonstrated in Chapter 3) may be significant in the pathogenesis of post-operative disease recurrence in susceptible individuals (IL-10 KO mouse). The histological changes were evident when the inflammatory cell (lymphocyte and neutrophil) infiltration of the peri-ileal or mesenteric fat analysis (11-point tissue injury score) was included.

The role of adipose tissues in inflammatory processes is a new and rapidly expanding research area. Adipose tissue is a source of soluble mediators called adipocytokines. These are predominantly produced by the adipocytes and exhibit immune properties.[9] Adipocytes have also been shown to produce the more traditional cytokines such as IL-6.[9] In addition, adipose tissue can have varying degrees of inflammatory cell infiltration. Macrophages are the predominant cell type infiltrating the adipose tissues, producing TNF and IL-6 and perhaps driving the adipose tissue mediated inflammatory responses.[9] The imbalance between the anti- and pro-inflammatory adipocytokines seen in obesity has been implicated in the pathogenesis of diseases such as diabetes mellitus, certain cancers and cardiovascular disease.[10] Although patients with CD tend to be malnourished and slim, the role of adipose tissue in

CD is being questioned due to a cardinal, gross pathological features of CD: "creeping fat". Creeping fat refers to the hypertrophied mesenteric fat that encompasses the diseased bowel.[11] Studies of creeping fat to date have shown increased production of adiponectin and leptin near bowel segments with active inflammation.[11, 12]

The presence of adiponectin and leptin provides evidence for an anti-inflammatory role of the creeping fat in CD as both adiponectin and leptin are anti-inflammatory mediators. The mesenteric fat changes demonstrated in our study were present at the neo-terminal ileum following ileocolonic anastomosis in 33% of animals at 6 weeks and 25% of animals at 15 weeks and they may in fact be significant. Future studies examining the release of adipocytokines and cytokines from the mesenteric fat at the surgical site will further elucidate the role of adipose tissue in post-operative disease recurrence.

In our study none of the WT mice developed colonic inflammation. In contrast, all the IL-KO mice had the classical histological findings of colonic inflammation previously described in this genetically engineered model.[5] As expected with the increasing age of the animals, the inflammation became more severe from the 6-week (13 week old animals) to the 15-week (22 week old animals) time point. The reduced tissue injury score at 15 weeks observed in mice after sham and ileocolonic anastomosis was not expected. A plausible explanation for this observation lies in bowel permeability. Increased bowel permeability is well described in both patients with CD and the IL-10 KO mouse and is thought to be a contributing factor in the disease pathogenesis by allowing increased bacterial translocation across the epithelial barrier.[13-16] A global decrease (improvement) in bowel permeability after bowel surgery has been reported in patients with CD undergoing ileocecal resection as well as in rodents.[17-19] In a rat model of short bowel syndrome resection of 75% of small bowel resulted in decreased permeability in the remaining bowel and this decrease was mediated by a serum factor that also decreased permeability in cultured rat intestinal epithelial monolayer.[18] Similar changes in permeability have been also described in IL-10 KO mice undergoing small bowel transection and anastomosis without resection (equivalent to our sham operation).[19] Perhaps an overall decrease in bowel permeability in the post-operative period results in decreased bacterial translocation across the colonic epithelium of IL-10 KO mice that ameliorates inflammatory response with subsequent reduction in tissue injury score. A drawback to this explanation is that the improved permeability reported in the above studies takes place in the immediate post-operative period (1 to 2 weeks) and we observed an improved tissue injury score at a more remote time point (15 weeks after surgery).[17-19] To clarify the role of permeability in the post-operative improvement of colonic tissue injury in IL-10 KO mice, it would be worthwhile in future experiments to assess bowel permeability at this remote time point.

As previously noted, TGF-β can be both beneficial and pathogenic in IBD. It can be beneficial by suppressing the over-active T-cell mediated immune response in CD and it plays a major role in wound healing after surgery.[20, 21] TGF-β can also be pathogenic by driving the differentiation of the naïve T cells towards the pro-inflammatory Th17 lineage, and by contributing to fibrosis that leads to bowel strictures.[22, 23] In Chapter 5 we demonstrated increased mucosal TGF-β mRNA in the IL-10 KO mice following the sham and ileocolonic anastomosis operations at 15 weeks after surgery. This elevation was the highest in the ileocolonic anastomosis group. In the present study we evaluated the terminal ileum segments for post-operative collagen deposition. We found collagen deposition to be increased in the WT and IL-10 KO mice at 6 weeks after sham and ileocolonic anastomosis operations. This is expected as part of normal tissue repair following bowel injury inflicted by surgery itself. At 15 weeks the collagen content decreased in the WT mice and remained close to unchanged in the IL-10 KO sham-operated mice, which is also expected due to scar remodeling. In contrast, in the IL-10 KO mice following ileocolonic anastomosis, the collagen deposition further increased from the 6-week to the 15-week time point.

The elevated mucosal TGF-β production (described in Chapter 5) coincides with increased collagen deposition within the neo-terminal ileum and may be a contributing factor in post-operative disease recurrence especially stricturing-type disease.

6.5. CONCLUSION

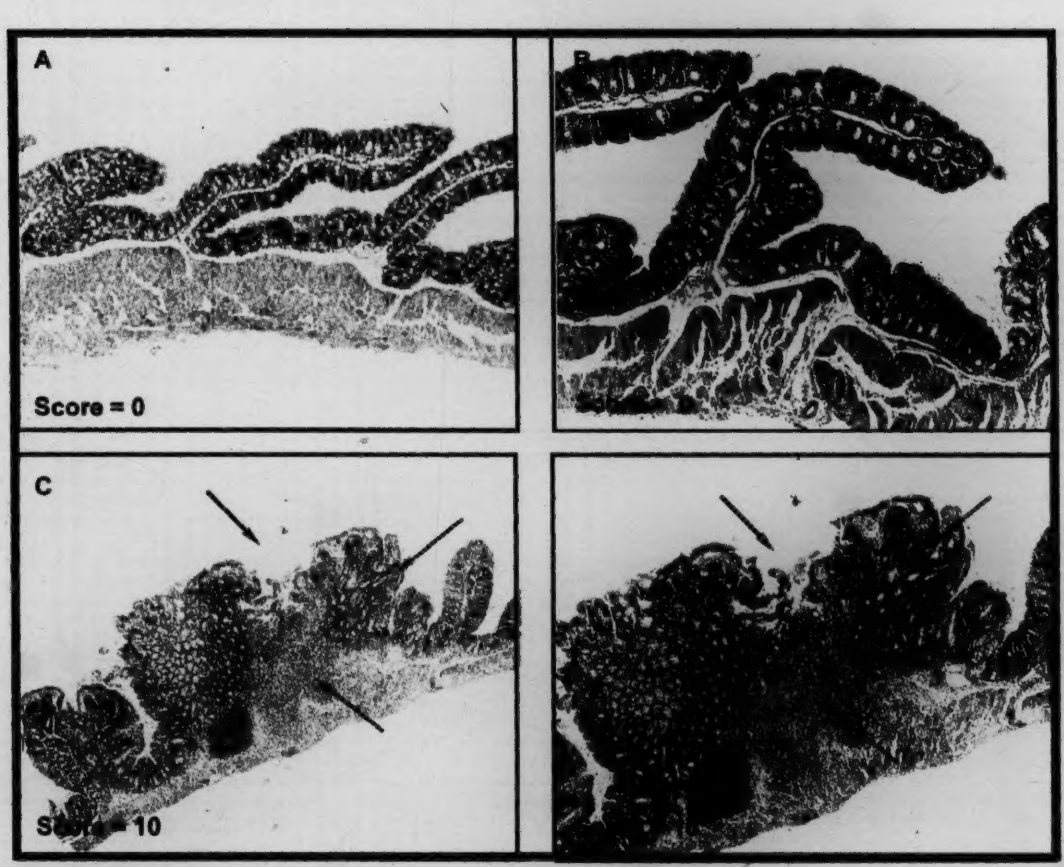
Our histological analysis did not reveal mucosal inflammatory changes in the neo-terminal ileum after ileocolonic anastomosis that are typical of patients with CD following a similar operation. We did, however demonstrate inflammatory infiltration of the mesenteric fat and increased collagen deposition in the neo-terminal ileum after IA in IL-10 KO. These findings provide valuable clues about the processes present in the post-operative bowel that is susceptible to inflammation (IL-10 KO mouse bowel) as there is limited understanding of the post-operative processes in this setting. Perhaps our findings can motivate and direct future studies in animal models and humans that will further expand out knowledge in this area.

Table 6.1. Histological injury scoring system

Characteristic	Description	Score
Enterocytes	Normal: rare intraepitheliala lymphocytes	0
	Mild: intraepithelial neutrophils	1
	Moderate: mucosal necrosis and/or luminal pus	2
	Severe: muscularis propria necrosis	3
Epithelial hyperplasia	Normal	0
	Mild	1
	Moderate	2
	Pseudopolyps	3
LP mononuclear infiltrate	Normal: 1 small lymphoid aggregate	0
	Slightly increased: >1 small aggregate	1
	Markedly increased: large aggregates and/or greatly increased single cells	2
LP neutrophil infiltrate	Normal: no extravascular presence	0
	Slightly increased: single neutrophils	1
	Markedly increased: cell aggregates	2

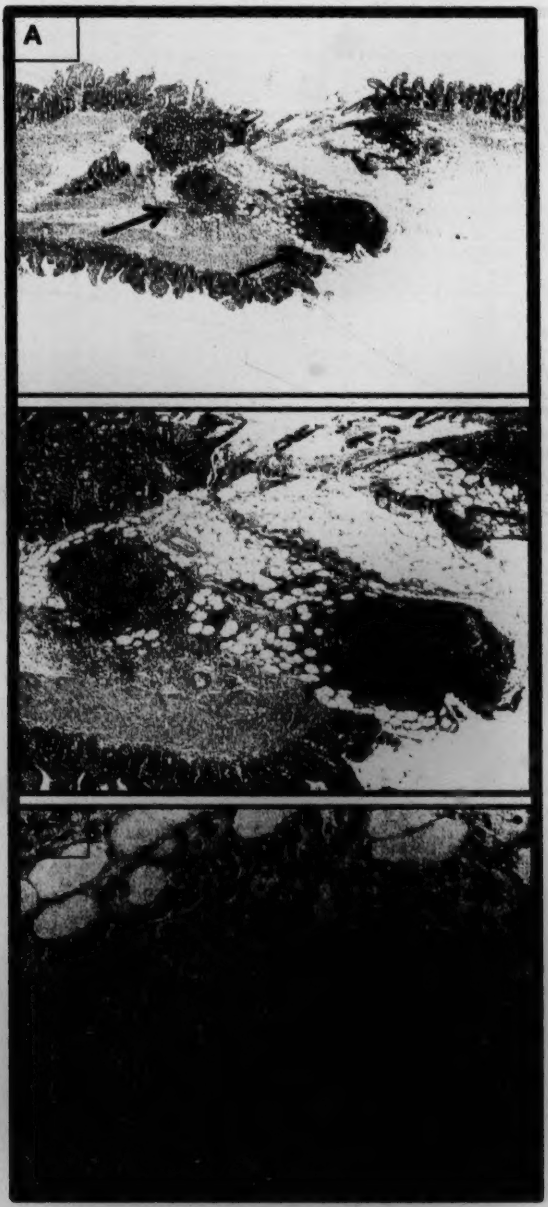
LP = lamina propria; no injury = 0; maximal injury = 10.

Figure 6.1. Histology of normal and inflamed mouse colon



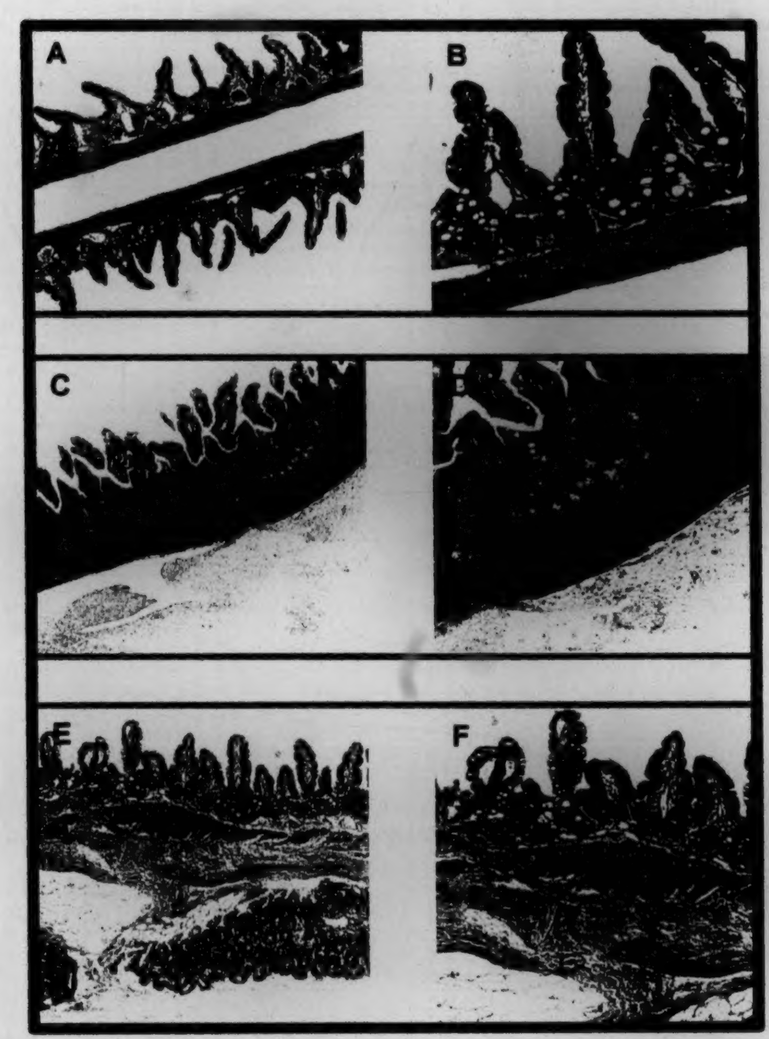
Hematoxylin and eosin stain of colon. **A & B.** Normal WT mouse colon 10X and 20X magnification respectively. **C & D.** IL-10 KO colon at 10X and 20X magnification respectively with maximal injury (score of 10): epithelial necrosis (black arrow), epithelial hyperplasia (red arrow) and lamina propria mononuclear and neutrophilic infiltration (blue arrow).

Figure 6.2. Peri-ileal fat inflammation



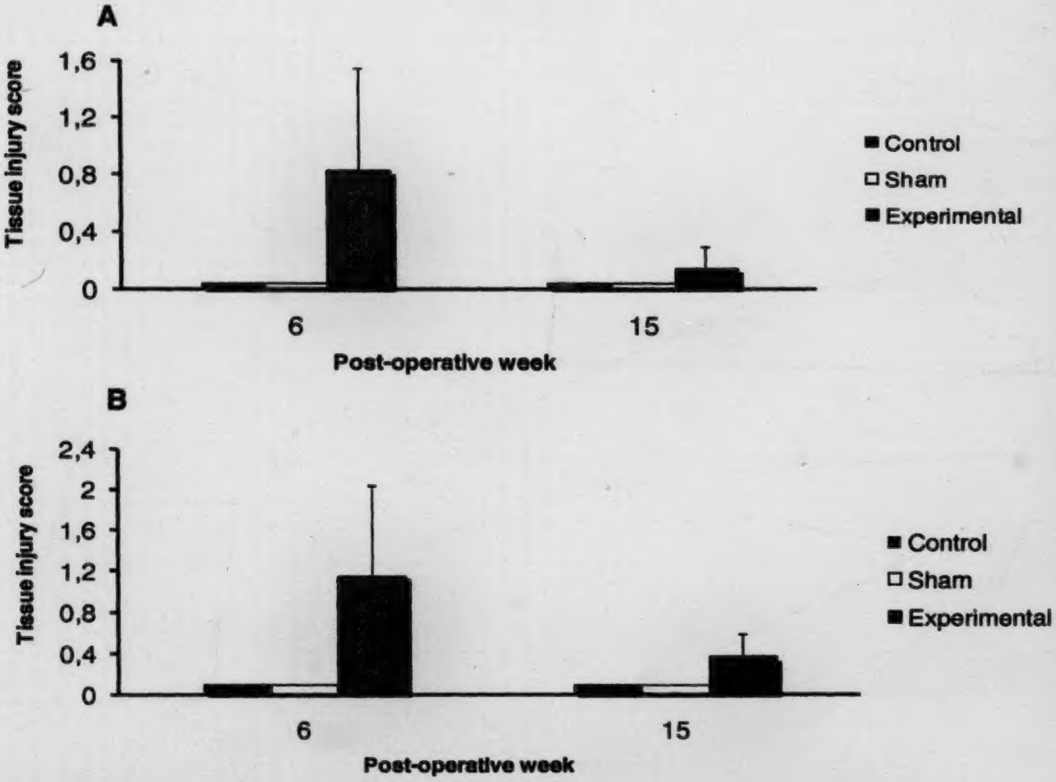
Hematoxylin and eosin stain of IL-10 KO mouse neo-terminal ileum after ileocolonic anastomosis with peri-ileal fat lymphocytic infiltration (black arrows) at **A** 10X maginifaction, **B** 20X magnification and **C** 40X magnification.

Figure 6.3. Collagen deposition score



Masson's Trichrome stain of mouse ileum with variable degree of collagen (in green) deposition. (A & B) score = 0 (10X and 20X magnification), (C & D) score = 1 (10X and 20X magnification), (E & F) score = 2 (10X and 20X magnification).

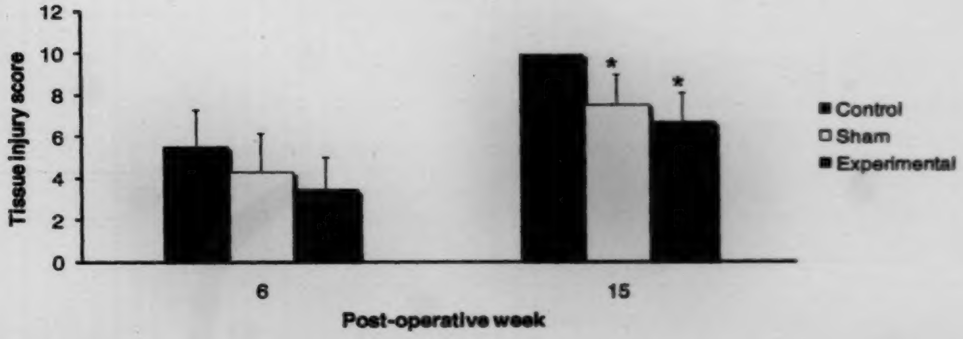
Figure 6.4. Ileal tissue injury score for IL-10 KO mice at 6 and 15 weeks after surgery



A. 10-point tissue injury score for IL-10 KO mice. There was a small but insignificant increasein animals the underwent ileocolonic anastomosis (experimental). **B.** 11-point tissue injury score for IL-10 KO mice. The observed increase in score in animasl after ileocolonic anstomosis (experimental) was not statistically significanr.

For each time point and group N = 6.

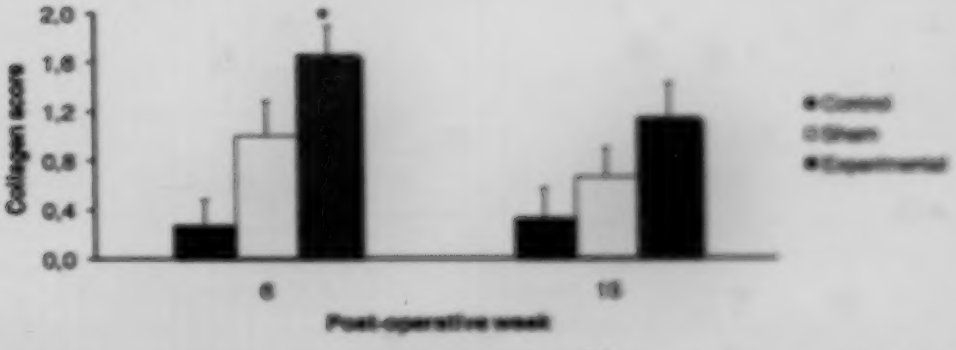
Figure 6.5. Colonic tissue injury score for IL-10 KO mice at 6 and 15 weeks after surgery



There was a statistically significant improvement in the colonic tissue injury score in IL-10 KO mice after sham and ileocolonic anastomosis (experimental) operations compared to IL-10 KO control groups at 15 weeks after surgery (* $p < 0.001$).

For each time point and group N = 6.

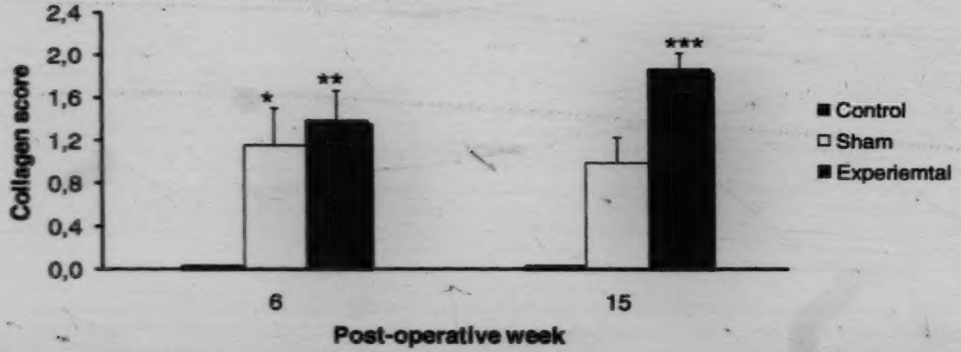
Figure 6.6. Ileal collagen deposition score for WT mice at 6 and 15 weeks after surgery



At 6 weeks after surgery there was a statistically significant increase in collagen content in the ileum of WT mice that underwent ileocolonic anastomosis (*p = 0.004). At the 15-week time point the collagen content was lower and no longer statistically significant. There was no statistically significant difference between sham and ileocolonic anastomosis groups at both time points.

For each time point and group N = 6.

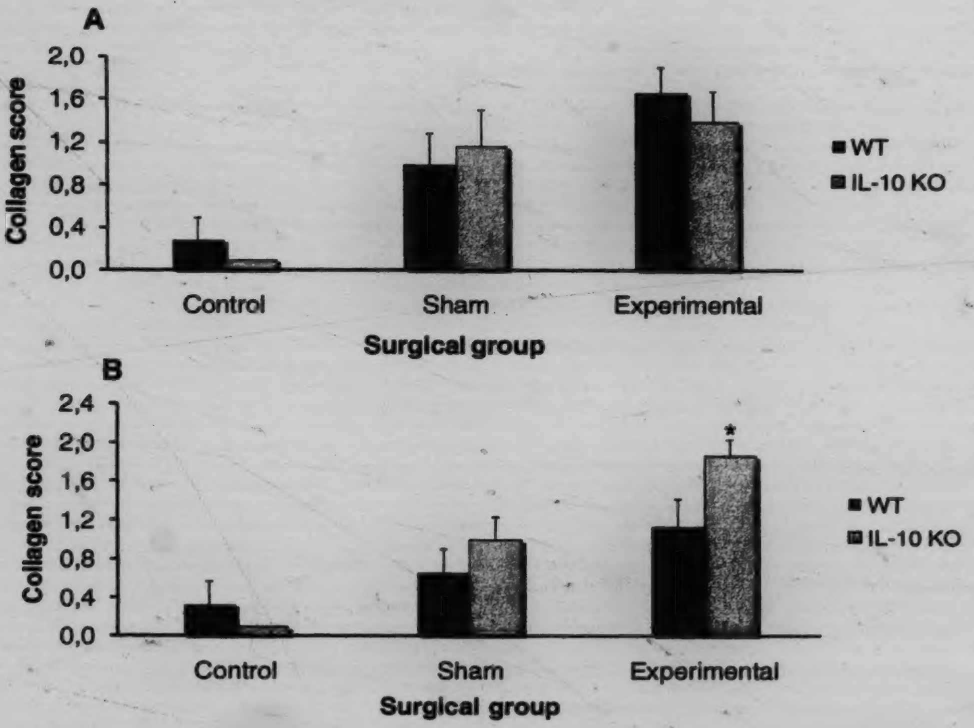
Figure 6.7. Ileal collagen deposition score for IL-10 KO mice at 6 and 15 weeks after surgery



At 6 weeks after surgery there was a statistically significant increase in collagen content in the ileum of IL-10 KO mice that underwent sham (*p = 0.004) and ileocolonic anastomosis (**p = 0.001). There was no statistically significant difference between the sham and ileocolonic anastomosis (experimental) groups at this time point. At 15 weeks after surgery collagen content was further increased in IL-10 KO mice following ileocolonic anastomosis, which was statistically significant when compared to control and sham-operated groups (***p < 0.010).

For each time point and group N = 6.

Figure 6.8. Ileal collagen deposition score for WT and IL-10 KO mice at 6 and 15 weeks after surgery



A. At 6 weeks after surgery collagen content within the ileum was elevated in sham and ileocolonic anastomosis (experimental) groups in both WT and IL-10 KO mice. There was no difference between the WT and IL-10 KO mice at the 6-week time point. **B.** At 15 weeks after surgery, the collagen score was statistically significantly higher in the IL-10 KO mice that underwent ileocolonic anastomosis than the WT mice after the same operation (* $p = 0.035$).

For each time point and group N = 6.

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Chapter 7. Putting the Pieces Together

Management of patients with CD after ileocecal resection poses a challenge to a clinician due to high clinical and surgical recurrence rates yet limited efficacious preventive measures. This challenge is further compounded by our lack of understanding of the post-operative pathogenesis that leads to almost universal and rapid disease recurrence at the anastomotic site and in the neo-terminal ileum. Based on our knowledge of the gastrointestinal anatomy, the technical aspects of ileocecal resection with ileocolonic anastomosis, and the underlying CD pathogenesis, we can speculate that ileocolonic anastomosis results in increased retrograde fecal flow into the terminal ileum causing changes in the endogenous bacterial flora of the small bowel. As described in previous chapters (Chapters 1 and 3), endogenous bacterial flora plays a pivotal role in bowel inflammation present in both CD patients and animal models of the disease.

To expand our knowledge in this area we undertook the task of developing a surgical animal model of post-operative CD and used this model to study bacterial flora, immune, functional and histological changes within the neo-terminal ileum. Using a well-established murine model of CD, the IL-10 KO mouse, we successfully reproduced ileocolonic anastomosis by constructing a wide-lumen side-to-side anastomosis, which is routinely performed in patients following ileocecal resection. The complications associated with our ileocolonic anastomosis were predominantly anastomotic leaks and obstructions leading to an acceptable mortality rate of 11%. Mice growth rates were not affected by the ileocolonic anastomosis. Using 16S rRNA PCR-DGGE followed by Dice

Similarity Coefficient calculation, we demonstrated that ileocolonic anastomosis results in increased similarity in the bacterial diversity shared by the colon and ileum. In IL-10 KO mice, ileocolonic anastomosis led to increased mucosal expression of TNF-α (pro-inflammatory cytokine) and TGF- β (anti-inflammatory). The elevated mucosal TGF- β in this particular group of animals (IL-10 KO with ileocolonic anastomosis) coincided with significant increase in collagen deposition at the surgical site. This finding is concordant with a previous report where elevated TGF- β in CD was associated with fibrosis and stricture formation.[1]

Our study also demonstrated that ileocolonic anastomosis and increased colonization of the neo-terminal ileum with colonic bacteria resulted in elevated expression of IFN-γ by splenocytes stimulated with endogenous bacterial antigens. This was present in both the WT and IL-10 KO mice at 15 weeks after surgery, suggesting that ileocolonic anastomosis leaves a lasting imprint in the immune system even in healthy individuals (WT mice). We speculate that the observed systemic response is the result of memory T cells formed in the peri-operative period. The transient breach of epithelial integrity during bowel surgery with subsequent bacterial translocation and increased bacterial antigenic exposure in the neo-terminal ileum can lead to formation of such memory T cells. IL-17 was also over-expressed by splenocytes in response to bacterial antigens but only by the IL-10 KO mice following ileocolonic anastomosis at the 15-week time point. The increase of IL-17 is perhaps a reflection of interleukin-10 deficiency in our model and may not be applicable to patients.

Bowel function, as assessed by glucose transport across the epithelium, was not changed by ileocolonic anastomosis. The neo-terminal ileum did not exhibit the classical mucosal injury that is present in the colons of IL-10 KO mice or in patients with post-operative recurrence, based on histological analysis. Some peri-ileal fat infiltration, however, were noted using histology in the IL-10 KO mice only following ileocolonic anastomosis, and this warrants further investigation in light of the evolving role of adipose tissue in inflammation. The lack of a reproducible and readily measured bowel injury in the neo-terminal ileum in our animals limits the utility of our model as a surrogate of ileocolonic anastomosis. Despite these negative results, based on our data and available literature we have been able to synthesize a theory that summarizes what we believe are the factors and post-operative changes that lead to disease recurrence. Our theory can be organized into answers to three simple questions: (1) Why? (2) Where? (3) When?

WHY

CD is a multi-factorial disease. The factors associated with CD can be broadly divided into three groups: (1) susceptible individual (genetics/immune system factors), (2) microbial factors and (3) environmental factors (Figure 7.1). The optimal combination and interaction of these factors results in bowel inflammation. The same three-group, multi-factorial approach can be applied in the explanation of the pathogenesis of disease recurrence following ileocecal resection (Figure 7.2). First, the underlying genetic susceptibility to develop CD bowel inflammation is unchanged by surgical manipulation. Furthermore, surgery adds an extra dimension to the already dysregulated immune system; the inflammatory response associated with wound healing. Second, ileocolonic anastomosis causes a disturbance in the endogenous microbial flora within the small bowel. In particular, the terminal ileum/neo-terminal ileum might be more sensitive to changes in bacterial antigen load given its unique and extensive immune network such as Peyer's patches and isolated lymphoid follicles within the lamina propria. Thirdly, smoking is a proven environmental risk factor for increased post-operative disease recurrence. The interaction between these factors determines the probability of disease recurrence.

WHERE

In the majority of patients undergoing ileocecal resection and ileocolonic anastomosis, the disease has been reported to recur at the anastomosis and extend into the neo-terminal ileum.[2] Recurrence within the neo-terminal ileum can be associated with changes in the microbial flora after the operation that removes the ileocecal valve. This has been demonstrated by our study (Chapter 3) as well by Neut *et al* in patients undergoing ileocecal resection.[3] As mentioned previously, the neo-terminal ileum may be sensitive to the introduction of new and perhaps increased number of bacterial antigens (both originating from the colon).

The high recurrence at the surgical anastomosis is the result of or associated with two key processes: fibrosis and inflammation. Fibrosis at the anastomosis results from wound healing in the presence of dysregulated immune system. The elevated mucosal production of TGF-β and subsequent increased collagen deposition demonstrated in our study supports this theory. The frequent free reflux of colonic contents past the anastomosis due to loss of the ileocecal valve, as well as probable anastomotic narrowing due to fibrosis causing stasis, both lead to increased bacterial colonization at the anastomosis. This bacterial colonization provides a constant stimulus for the inflammatory response. It is also of paramount importance to understand that the two processes, fibrosis and inflammation are not distinct entities but most likely augment each other by sharing cells and molecular mediators (Figure 7.3). For instance, TGF-β production is increased at the anastomosis after surgery. IL-6, a pro-inflammatory cytokine also necessary for wound healing, has been shown to be elevated at the anastomosis in patients with CD following ileocecal resection, yet is absent in bowel remote to the anastomosis (in the rectum), and is predictive of disease recurrence.[4] Perhaps, the right concentration of TGF-β and IL-6 produced as part of wound healing and in response to increased bacterial antigens in the neo-terminal ileum induces the pro-inflammatory Th17 lineage, suggesting that local and overlapping inflammatory responses may lead to disease recurrence.

WHEN

A large number of patients have evidence of disease recurrence on endoscopy in the early post-operative period (within the first year).[5] The rapidity of post-operative recurrence is best explained by a synergy of post-operative wound healing and immune response to altered bacterial antigens at the anastomosis and within the neo-terminal ileum in a susceptible individual (dysregulated immune system). In addition, surgical manipulation of the bowel such as an enterotomy most likely generates memory T cells. These cells can persist within an individual and mount a faster and more aggressive response on repeated encounters with the endogenous flora, which is most likely increased in the neo-terminal ileum after ileocolonic anastomosis. In summary, there are multiple immune responses in the post-operative period that set up a complex network of local and systemic immune mediators such as cytokines (Figure 7.3). Perhaps the overlap and interaction between these cytokines results in an amplified and exaggerated Th1 and Th17 mediated bowel injury that explains the rapid and aggressive recurrence after ileocecal resection.

The findings presented in this thesis add valuable information to the current knowledge of post-operative changes, yet the understanding of the pathogenesis of post-operative recurrence remains incomplete. Many gaps remain in our understanding of the mechanisms involved in recurrence, which means there are countless opportunities for new and exciting discoveries. The expansion of our knowledge and more importantly, the well being of the many patients with CD faced with ileocecal resection, depend on continuation of animal model studies such as ours and the extension of such studies to humans.

Figure 7.1. Factors in Crohn's disease

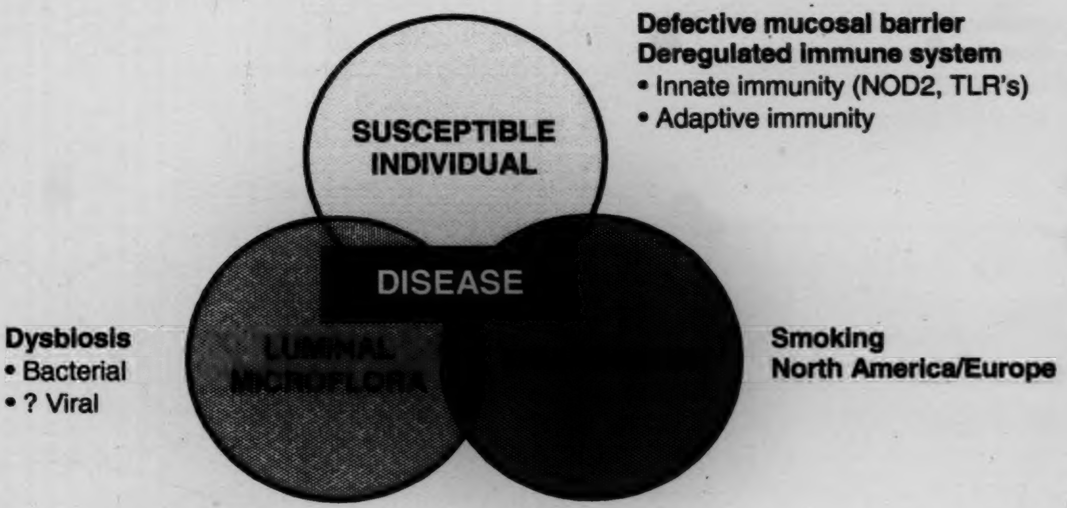

Defective mucosal barrier
Deregulated immune system
• Innate immunity (NOD2, TLR's)
• Adaptive immunity
SUSCEPTIBLE INDIVIDUAL
DISEASE
Dysbiosis
• Bacterial
• ? Viral
LUMINAL MICROFLORA
Smoking
North America/Europe

Figure 7.2. Factors in post-operative Crohn's disease recurrence

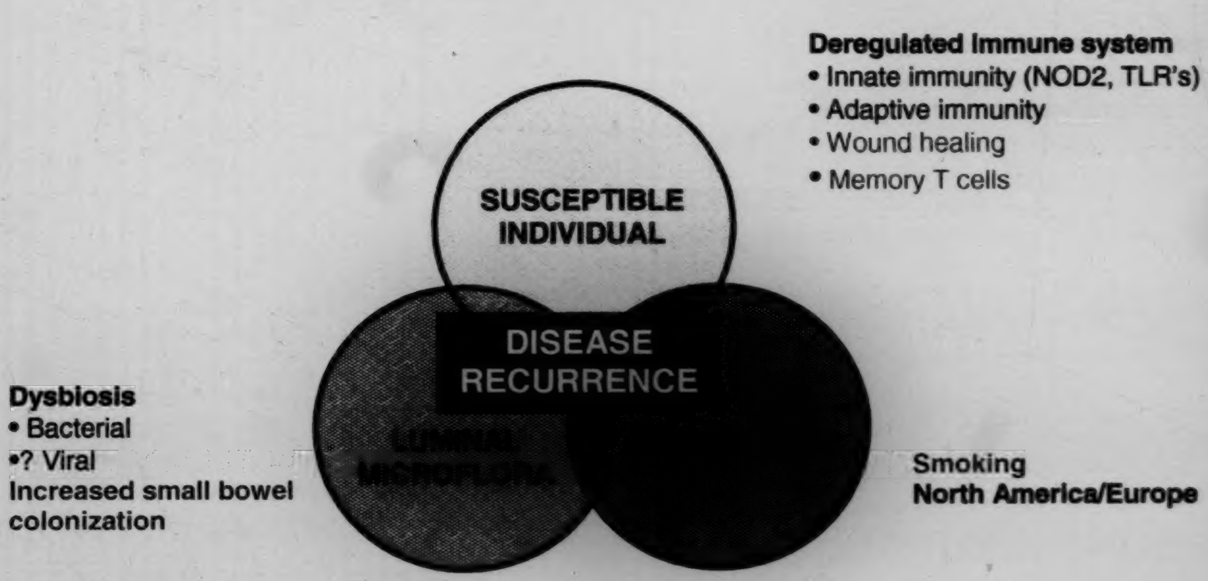
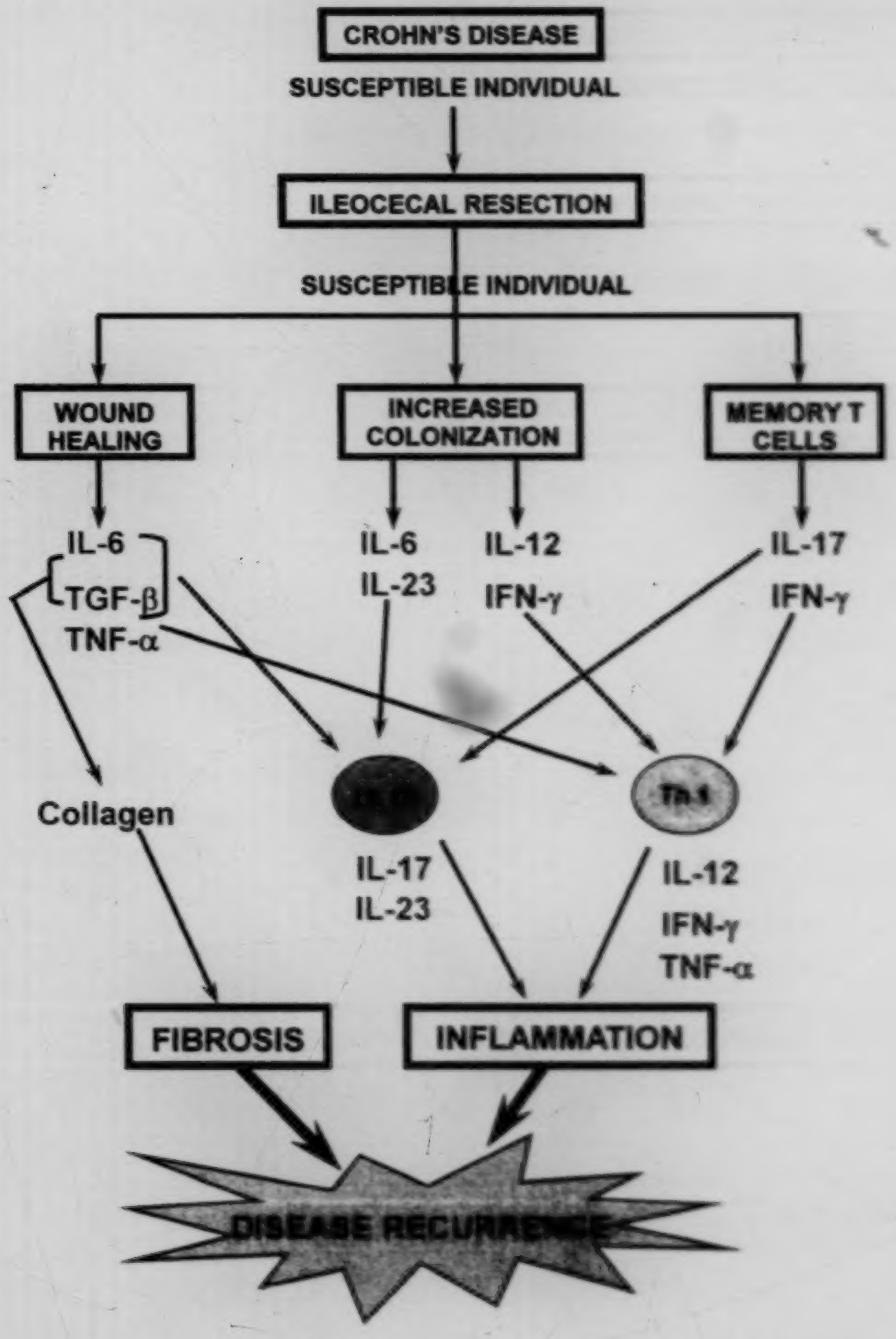

Deregulated immune system
• Innate immunity (NOD2, TLR's)
• Adaptive immunity
• Wound healing
• Memory T cells
SUSCEPTIBLE INDIVIDUAL
DISEASE RECURRENCE
Dysbiosis
• Bacterial
•? Viral
Increased small bowel colonization
LUMINAL MICROFLORA
Smoking
North America/Europe

Figure 7.3. Pathogenesis of post-operative recurrence of Crohn's disease


CROHN'S DISEASE
SUSCEPTIBLE INDIVIDUAL
ILEOCECAL RESECTION
SUSCEPTIBLE INDIVIDUAL
WOUND HEALING
INCREASED COLONIZATION
MEMORY T CELLS
IL-6
TGF-β
TNF-α
IL-6
IL-23
IL-12
IFN-γ
IL-17
IFN-γ
Collagen
Th1
IL-17
IL-23
IL-12
IFN-γ
TNF-α
FIBROSIS
INFLAMMATION
DISEASE RECURRENCE

7.1. REFERENCES

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